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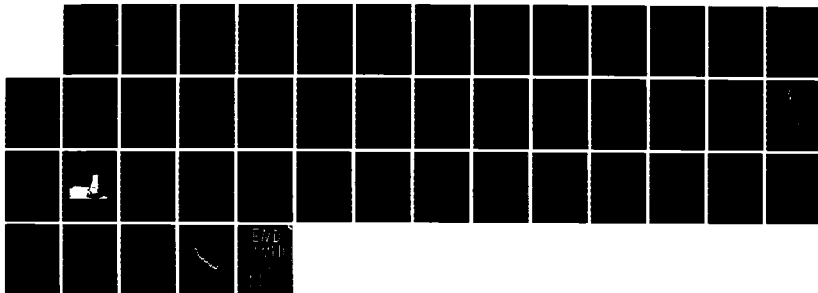
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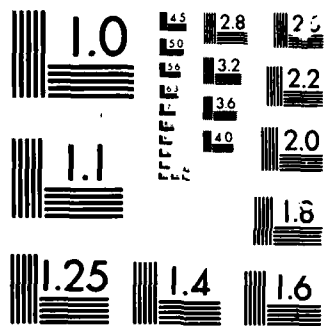
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## Advantages of the Gas Exchange Approach to Microbiological Studies

P. J. HANNAN AND D. S. JONES

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) <p>Studies of the effects of various chemical or physical stimuli on the growth rates of microorganisms generally involve some measure of biomass. In the case of algae, one might measure the cell number, the chlorophyll concentration, the fluorescence, or the wet weight of the culture as a function of time. Each such measurement requires a sampling of the culture which could be a disturbing factor in the system being measured. Another disadvantage of these traditional measurements is that the times required for significant change to take place in the culture might be hours or days; furthermore, growth rate measurements calculated from the data must be based on the assumption that during the time between measurements the growth rate was constant.</p> <p>This report describes another approach to the problem. It consists simply of monitoring the O<sub>2</sub> or CO<sub>2</sub> concentration of an air stream passing through the system (the "gas exchange" method), and it has several distinct advantages: 1) there is no need to take samples of the culture, 2) each measurement is a rate measurement and indicates the performance of the culture at that very moment, and 3) transitory changes in growth rates are readily detected. Examples are given of studies made previously with this method at NRL. Also the possible value of these methods in a study of the corrosion susceptibility of alloys is described.</p>				
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## ADVANTAGES OF THE GAS EXCHANGE APPROACH TO MICROBIOLOGICAL STUDIES

### I. INTRODUCTION

The purpose of this report is to point out a unique aspect of a particular biological measurement and relate it to possible future studies at the Naval Research Laboratory. The methodology is relatively simple but it has not been used to the extent that its potential warrants.

The technique consists in monitoring, on a continuing basis, the  $\text{CO}_2$  (or  $\text{O}_2$ ) content of an air stream following its passage through a culture of microorganisms. If the air flow rate and the temperature of the liquid are constant, then a comparison of the input and exit gases provides an instantaneous measure of the growth rate of the culture. Transient changes in growth rates, resulting from the imposition of various stimuli, can be reflected in changes in gas composition; by choosing an organism which is particularly susceptible to a certain stimulus, the technique can have extreme sensitivity. This report will describe the research done previously at NRL which illustrates the rapid sensing of changes in growth rates based on gas exchange. Algal cultures have been the subject of these studies and in certain instances both  $\text{CO}_2$  and  $\text{O}_2$  measurements have been made; the same techniques could be used for studies of other microorganisms provided they have a sufficiently fast growth rate. An inherent advantage of  $\text{CO}_2$  measurements is that they can be made against a background of atmospheric  $\text{CO}_2$  concentrations which are several orders of magnitude less than  $\text{O}_2$  concentrations.

Traditional measurements of microorganism growth rates can be based on actual counts of cells, or analyses for adenosine triphosphate (ATP), chlorophyll, or other products, at various intervals of time. The time between measurements must be sufficient to allow significant changes to take place in the parameter being measured; small differences between large numbers are often encountered. By contrast, each gas exchange measurement is a rate measurement and the problem of a large background can be minimized. To illustrate the point, consider a hypothetical algal culture being aerated at a constant rate and diluted with fresh culture medium at a constant rate. The system comes to equilibrium with a fixed cell density because the cells being washed out are replaced quantitatively with new cells, and the culture is producing  $\text{O}_2$  at a constant rate. Now, suppose the aeration and dilution rates are constant but the

growth rate is reduced 10% (perhaps by a lower light intensity). Figure 1 depicts the gradual change in cell concentration which does not come to a new equilibrium until 10 hours have elapsed, whereas the new gas exchange rate is stabilized in a matter of minutes. The time required for the new gaseous equilibrium depends partially on the flow rate. For CO<sub>2</sub> equilibria the salinity is an important factor, also, which is covered later in this report. The essential point is that the gas exchange approach tells, in a matter of minutes, what would require hours if biomass were being measured. An assumption regarding comparability between O<sub>2</sub> production and biomass production is made for the purpose of the illustration in Figure 1; the authors are aware of the inaccuracy but prefer to make the case in the simplest form at this point.

The gas exchange approach has been used extensively at the Naval Research Laboratory in studies with the freshwater alga, Chlorella pyrenoidosa sorokiniana. In the paragraphs following, data will be presented to show the reliability of such measurements and the speed with which they reflect changes in culture conditions such as light intensity, CO<sub>2</sub> input rate, pressure, or the presence of toxic alloys. Interest in the gas exchange phenomenon began here with studies concerning the feasibility of providing oxygen for nuclear submarines by the use of mass cultures of algae. Many types of culture apparatus were used, each designed for a specific purpose. The final report on these studies stressed the reliability of the system but pointed out its impracticality because of the exorbitant electrical energy requirement (Hannan, Shuler, and Patouillet, 1963).

## II. MATERIALS AND METHODS

### Organism

Chlorella pyrenoidosa 7-11-05 (later given the sub-species name of sorokiniana) was isolated initially from a Texas swamp, and cultures were obtained from Dr. Constantine Sorokin while he was on the faculty of the University of Maryland. Its high growth rate, with a doubling time of only two hours under ideal conditions, made it particularly attractive for the purpose at hand (Sorokin and Myers, 1954). The organism is roughly circular with a diameter of approximately 5 microns, and has a temperature optimum of 37-39°C. Sterile conditions were not maintained, and tap water was used in the preparation of the medium (Table 1).

### Culture Devices

A. Six-lamp unit. Many experiments were performed with the large culture apparatus shown in Figure 2. It is described in detail elsewhere (Hannan and Patouillet, 1963 A), but the essential features were temperature control within 2°C, high



light intensities provided by six GE Quartzline lamps oriented vertically in the six liters of culture medium, rapid stirring, controlled rates of dilution with fresh culture medium, close control over the concentration of  $\text{CO}_2$  in the input air, and the rate of air input. Air was bubbled through the culture at rates up to 4 liters/min and allowed to escape through ports in the top of the unit. Sampling of the effluent air was by means of a small pump connected to a narrow-bore nylon tube which extended into the air space above the algal culture; the output of the pump (400 cc/min) was only a fraction of the air input rate to the system, therefore the effluent from the pump consisted solely of air that had passed through the culture. This six-lamp unit was used in the experiments described subsequently concerning the effects of light intensity,  $\text{CO}_2$  input rate, urea, and the 'Matthern' medium.\* It was used as the source of cells for experiments concerning the effects of pressure, and the toxicity of alloys, each of which required a different type of culture apparatus.

B. Pressure Unit. This unit (Figure 3) was made from glass pipe sealed with rubber O-rings into blocks of lucite, and contained two annular spaces around a vertically oriented Quartzline lamp. Cooling water flowed through the annulus nearest the light, and the algal culture was pumped through the outer annulus by a gear pump. A constant pressure was maintained within the culture chamber by manipulating stainless steel needle valves which controlled the input and exits of the air/ $\text{CO}_2$  mixtures used. The  $\text{CO}_2$  input rates (i.e., the product of the air flow rate times the  $\text{CO}_2$  concentration) could be varied by changing the flow rate of a given air composition, or by changing to a different  $\text{CO}_2$ /air mixture. Pressure cylinders of particular  $\text{CO}_2$ /air mixtures were used in this study to assure constant  $\text{CO}_2$  concentrations throughout an experiment. In these experiments, there was no dilution of the cultures with fresh medium. A complete description of the apparatus and results can be found elsewhere (Hannan, 1964).

C. Toxicity Unit. Various experiments were performed with this unit but the emphasis here will be placed on the experiments concerning the potential toxicities of various metal alloys. The unit used in this study (Figure 4) had two annular spaces surrounding a vertical Quartzline lamp, the inner containing cooling water and the outer containing the algal culture which was circulated by a gear pump. The outer shell of the unit was made of #304 stainless steel which, in turn, was surrounded by an annular space through which cooling water was circulated on demand from a thermoregulator extending into the culture. At the top of the annular space for the culture was an

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\*See page 6

O-ring seal through which a 3.18 mm (1/8") inch diameter rod could be extended, the rod being representative of a particular alloy to be studied. In the initial phase of an experiment, a 350 ml portion of a fresh algal culture taken from the six-lamp unit was placed in the apparatus and its O<sub>2</sub> production measured until a steady state had been achieved. Then the O-ring seal in the top of the unit was loosened slightly to permit lowering the rod into the culture, and O<sub>2</sub> production measurements were continued. The length of rod in the culture was 20.3 cm (8"). The results are described in the report by Hannan and Patouillet (1967). There was no dilution of the culture with fresh medium during these experiments.

#### Airflow Rate Measurements and Gas Analyses

Flowmeters containing either a Pyrex or steel ball were used, depending on the flow rate being measured; the meters used were products of either Ace Glass or the Fischer and Porter Co. Air leaving the culture chamber was first passed through a bubbler\* and then a Drierite (anhydrous CaSO<sub>4</sub>) column to remove water vapor, before passing through the flowmeter.

The O<sub>2</sub> analyzer was a Model F-3 (Beckman Instruments, CA) with two scales, 0-25% and 20-25%, the latter being the one generally used because estimates of O<sub>2</sub> concentrations could be made to 0.01%. The CO<sub>2</sub> analyzer was the product of Mine Safety Appliances (Pittsburgh, PA), with a single 0-2% range which was later modified to extend to 4%. Zero settings of each instrument were made with N<sub>2</sub> passing through; the O<sub>2</sub> analyzer was spanned with air (assumed to be 20.95% O<sub>2</sub>) and the CO<sub>2</sub> analyzer was calibrated with standard gases whose CO<sub>2</sub> content had been determined earlier with a modified Orsat apparatus. With this particular apparatus, CO<sub>2</sub> analyses were reproducible within 0.02%.

In order for meaningful CO<sub>2</sub> uptake measurements to be made, it was necessary to control the input CO<sub>2</sub> concentration closely. Because the flow rates of air through the six-lamp unit were so high, it was not practical to use pre-mixed air from cylinders. Instead, a manifold was constructed to connect four CO<sub>2</sub> cylinders to a mixing system made at NRL (Bultman and Hannan, 1967) in which compressed air from the laboratory was mixed with CO<sub>2</sub> at a controlled rate by means of a needle valve on the CO<sub>2</sub> inlet. With this equipment, it was possible to maintain a CO<sub>2</sub> concentration within several hundredths of a percent for weeks at a time.

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\*A small suction flask fitted with a rubber stopper holding a glass T; the lower leg of the T extended into a dilute acid solution. Air flowing through the exit of the T was controlled by a screw clamp to give the desired flow rate into the analyzer.

A particular characteristic of this unit was that the concentration of  $\text{CO}_2$  was not affected by the rate at which the air was tapped off the mixing system.

For experiments conducted with the pressure unit or the toxicity unit, the  $\text{CO}_2$ /air mixtures were taken from cylinders containing known concentrations of  $\text{CO}_2$ .

The time required for the gas analyzers to equilibrate with an air stream depended on the flow rate of air, the volume of connector tubing and desiccant in the train of equipment leading to the analyzers, and the type of algal culture being used; the complication attendant with  $\text{CO}_2$  uptake measurements concerned only high salinity, which is discussed at length later in this report. For the experiments with the fresh water alga, at a flow rate of 100 cc air/min, about eight minutes was required for the meters to reflect accurately the composition of the air; with the six-lamp unit, the higher flow rates of air required even less time for equilibrium.

### III. RESULTS

#### Effect of Light Intensity on $\text{CO}_2$ Uptake and $\text{O}_2$ Production

Light intensity within the six-lamp unit could be changed quickly since one large variac controlled the voltage on the lamps. Figure 5 represents the change in  $\text{CO}_2$  uptake and  $\text{O}_2$  production with a change in voltage; with 200 volts on the lamps the  $\text{O}_2$  production was 3,400 cc/hr but when the voltage was dropped to 175 the  $\text{O}_2$  production became approximately 2,700. The new equilibrium value of the  $\text{O}_2$  production was apparent in only two minutes, with a little more time required for the  $\text{CO}_2$  absorption to reach a new steady state. Several factors played roles in the times required for new equilibria to be established: 1) The high air flow rate through the culture was effective in stripping the dissolved  $\text{O}_2$  and  $\text{CO}_2$  from the culture and, 2) Equilibrium between gaseous  $\text{CO}_2$  and the liquid culture probably required more time than that of the  $\text{O}_2$  because of the salinity of the culture. This factor is explored in more detail later in this report. When the voltage on the lamps was restored to 200 the original  $\text{O}_2$  and  $\text{CO}_2$  values returned, once again with a slight delay in the case of the  $\text{CO}_2$ .

It is possible, of course, to demonstrate a rapid change in  $\text{O}_2$  production with change in light intensity with the use of a polarograph. With a heavy deposit of algal cells placed directly on a polarographic electrode, changes in oxygen tension can be monitored with time but such a system does not provide the flexibility of studies which is possible with the gas exchange approach.

### Effect of CO<sub>2</sub> Input Rate

It had been shown earlier that the rate of O<sub>2</sub> production depended on the CO<sub>2</sub> input rate, i.e., the product of the CO<sub>2</sub> concentration times the flow rate. This can be detected almost immediately by the gas exchange approach, as shown by the plot in Figure 6. With a 3.8% CO<sub>2</sub> concentration in an air flow at 2,200 cc/min, the CO<sub>2</sub> input rate was 5,020 cc/hr and the O<sub>2</sub> production rate was 3,380 cc/hr. By reducing the air flow rate to 1,743 cc/min the CO<sub>2</sub> input rate became 3,975 and the O<sub>2</sub> production dropped to 3,200. When the flow rate was increased again, the O<sub>2</sub> production rose above the expected value and stayed there for approximately a half-hour before resuming the normal value for those conditions. This was probably a consequence of the photosynthetic potential developed in the cells when they were in a CO<sub>2</sub>-deficient status, resulting in a temporarily enhanced productivity when more CO<sub>2</sub> became available. This transient condition would not likely be detectable by monitoring cell counts or chlorophyll content.

### Effect of Additional Urea on Gas Exchange Rates

Included among the attempts to maximize O<sub>2</sub> production was an experiment to determine the effect of an increased nitrogen concentration in the culture medium. The six-lamp culture unit had been at equilibrium, while being diluted with fresh culture medium at 15%/hr, when solid urea was added in an amount calculated to double the concentration in the culture medium. Because of the fast dissolution of urea in this rapidly stirred suspension, the change in urea concentration was probably almost immediate.

The effect of this urea addition on gas exchange is illustrated in Figure 7. The gyrations in CO<sub>2</sub> uptake and O<sub>2</sub> production are not understood but the major point to be stressed is that changes in packed cell volume (which were included in this study) were minor compared to the large changes in gas exchange. Within a four-hour period, the CO<sub>2</sub> absorption had decreased by 7.9% and then increased by 3.9% during which time the packed cell volume had shown a slight steady increase. The last reading of the day indicated a marked decline in both CO<sub>2</sub> uptake and O<sub>2</sub> production but by the next morning the cells had recovered and their production was normal. Dilution with fresh medium had continued overnight so that there was a dual effect on the system, i.e., a gradual washout of the excess urea and probably an acclimation, with time, of the cells to their changed environment.

### Adaptation to a More Concentrated Culture Medium

In another experiment to maximize O<sub>2</sub> production by the Chlorella culture, a concentrated culture medium (devised by Dr. Robert Matthern at the Quartermaster Laboratories in

Chicago) was substituted for the normal medium. Within less than four hours, when the dilution rate was 15% hr, the  $O_2$  production had decreased by one-third and within 20 hours it had dropped by almost one-half (Figure 8) at which point the dilution was stopped lest all the cells be washed out. Ultimately, the dilution was resumed but only at 3.3%/hr, then at 6.5% and finally 13% on the fourth day of the experiment. Changes in the gas exchange characteristics of the culture had provided an early warning of its potential demise in a manner that could not have been done with the cell-counting method. It was interesting that the culture finally achieved the normal gas exchange values with this concentrated medium and, also, that it maintained them when it was switched back to the regularly used medium.

#### Effect of Pressure on $O_2$ Production

A factor to be considered in an algal culture unit for submarine use would be the relatively low ambient concentrations of  $CO_2$  compared to those used in our experiments. The premise was that pressurization would increase  $CO_2$  solubility and, therefore, enhance  $O_2$  production. The equipment has already been described and is pictured in Figure 3.  $CO_2$ /air mixtures were supplied from cylinders having fixed  $CO_2$  concentrations and for each 10 psi increment studied, there were  $CO_2$  input rates of 30-, 60-, and 100% saturation at the light intensity used. A given culture was used for only one pressure study lest there be some residual effect of pressure, so that for each pressure condition there were three different batches of cells used.

The results are summarized in Figure 9. The  $O_2$  production, at time zero, represented the steady state value for the culture at atmospheric pressure with that particular  $CO_2$  input rate. With the imposition of pressure the  $O_2$  production generally changed and, except for the highest  $CO_2$  input rates used, there tended to be a temporary increase in  $O_2$  production followed by a decline; the higher the pressure imposed, the more drastic the decline in  $O_2$  production. Experiments with cell-free solutions showed that within 15 minutes a steady state had been established between the liquid and the  $O_2$  and  $CO_2$  in the air stream, therefore the  $O_2$  production and  $CO_2$  absorption values shown after that time were the result of biological action of the algal cells and not merely attributable to physical effects.

There was a possibility that this reduced  $O_2$  production was related to an increase in  $O_2$  tension in the liquid because the literature contains references to inhibitions obtained with algal growths subjected to high  $O_2$  concentrations. To test this hypothesis, a special mixture of  $CO_2$  (2.49%) and  $O_2$  (6.84%) in  $N_2$  was provided at a single  $CO_2$  input rate of 1,716 cc/hr, and at 10 to 50 psi. A plot of  $O_2$  production vs.

time under pressure was similar to that when air/CO<sub>2</sub> mixtures were used, indicating the oxygen tension was not a significant factor in the inhibitions described.

The principal point to be made is that the gas exchange rates changed quickly under pressure. It would not be possible to determine the magnitude of these transient changes by monitoring cell counts or other measures of biomass because there would need to be a constant productivity over a period of several hours to establish a definite relationship between gas exchange and biomass production.

#### Toxicity of Metal Alloys to Chlorella

It is known that certain alloys are toxic, based on the comparative growth rates of algal cultures in the presence of these alloys (Dyer and Richardson, 1962). To build a large algal gas exchanger for submarine use would require that non-toxic structural materials be used, and the intent of this study was to explore further the range of options available; instead of the traditional approach used in the Dyer and Richardson study, the procedure was to introduce test alloys into a growing algal culture and determine their effect on the culture by monitoring gas exchange.

Each experiment consisted in establishing the O<sub>2</sub> production of a batch culture of Chlorella, and then inserting into it a 3.8 mm diameter rod while continuing to monitor the O<sub>2</sub> production. Figure 10 represents the data obtained with a rod made of #304 stainless steel; within 15 minutes after the insertion of the rod the productivity dropped to a lower plateau and remained there. This curve typifies the results obtained with a variety of alloys. Complete details of this study can be found elsewhere (Hannan and Patouillet, 1967) but the principal finding was that the surface properties of an alloy, and not its bulk composition, determine whether it will exert a toxic action on a culture. Many experiments were performed with the rod that was the subject of Figure 10, and eventually its toxicity was lost which would indicate that some leachable component had finally disappeared entirely from the surface. It was conceivable that this component(s) was being released from a grain boundary; to test this hypothesis, the specimen was stretched 1% of its length in a tensile test machine and then assayed again with a Chlorella culture. The toxicity of the rod was restored, the presumption being that a new grain boundary had been exposed from which toxicants could be released.

The most compelling evidence that the bulk composition was not the determinant in these toxicity studies was obtained with a 182.9 cm length of #316 stainless steel which was cut into seven 25.4 cm specimens tested. Four showed evidence of toxicity (ranging from 8 to 15% inhibition) while three had essentially no effect. Also pertinent to this theme of surface

composition as the determinant in activity were the results obtained with a brass rod which was assayed on four occasions after being buffed. The results ranged from a slight enhancement in productivity to 24% inhibition.

No provisions were made in this culture unit for continuous dilution with fresh medium, so it was not possible to determine whether the inhibitory effects might be of short or long duration. Beyond a period of about four hours, there would be the possibility that a reduction of  $O_2$  production would be the result of a nutrient deficiency which could be misinterpreted as a toxic effect.

#### Reliability of Algal Cultures

In the preceding paragraphs, stress was placed on the speed with which changes in gas exchange rates can be detected following the addition of a stimulus to the cultures. The credibility of such data depends on the reliability of the method and examples will be shown here of a high degree of reproducibility obtained with different types of algal cultures used in different ways; the first concerns data on  $O_2$  production by the fresh water Chlorella, and the second relates to growth rate studies (chlorophyll production) made with the marine diatom, Phaeodactylum tricornutum.

The reliability of the six-lamp unit was determined in a series of measurements made over a five-day period in which the culture was operated at a fixed  $CO_2$  input rate and a fixed dilution rate, with eight measurements of  $O_2$  production and  $CO_2$  uptake made each day. For sixteen hours out of each day, there was no one in attendance. For the gas exchange measurements, it was necessary to calibrate the  $O_2$  and  $CO_2$  analyzers and connect the appropriate inlet and outlet sources to them at the proper times. The results are included in the report by Hannan and Patouillet (1963) and summarized in Table 2. The high standard deviation in  $O_2$  production on the first day indicated the gradual acclimation of the culture to the conditions established, since the culture had been in the refrigerator during the weekend prior to the test. By the 5th day of the test, however, with an average  $O_2$  production rate of  $3,638 \text{ cc hr}^{-1}$  the standard deviation was only 24, amounting to an error of less than 1%. This discrepancy could be caused by an error of only 0.01% in reading the  $O_2$  analyzer, and it was calibrated only in tenths.

The second example of reliability concerned a study of growth rates obtained with Phaeodactylum tricornutum, based on fluorescence measurements made after 20- and 44-hours of incubation. In these experiments, a standard toxicant was used, i.e., 3.3 parts per billion Hg (as  $HgCl_2$ ). Over a period of several months there were many experiments performed with a large batch of aged synthetic seawater, all performed with batch

cultures used in a definite regimen. The average inhibition of growth was 35% by the Hg, with a standard deviation of 4.3. This is considered an exceptionally low standard deviation for results with batch cultures used at different times. The sensitivity of the procedure is dramatized also when results are compared with a parallel study in which another batch of synthetic seawater was the base; here the inhibition with Hg was only 23%. While distilled water and C.P. chemicals were used for the preparation of both batches of seawater, there obviously was a difference in the results obtained. These experiments are described in detail in the report by Hannan and Patouillet (1979).

#### Limitations of Gas Exchange Measurements with Water of High Salinity

The equilibration of CO<sub>2</sub> with water is rather rapid when the salinity is low, such as with the culture medium for Chlorella. With high salinities, however, such as with seawater, the equilibration rate is so slow that gas exchange measurements are not possible. The question then arises as to the maximum salinity with which meaningful CO<sub>2</sub> exchange measurements can be made.

To determine this, an apparatus was constructed which consisted of a long glass tube filled with water through which a known concentration of CO<sub>2</sub> in air was bubbled until the water was saturated, i.e., the composition of the effluent air was the same as the input. Then air of a different CO<sub>2</sub> concentration was introduced and the time required for equilibration was measured. In these experiments, several factors were kept constant; the air flow rate was 10 cc/min, the bubble diameter was 5 mm, and the average temperature was 22.44°C with a maximum variation of 0.06°C. The CO<sub>2</sub> concentrations used were 0- and 346 ppm. Four salinities were used, viz. 25-, 50-, 75-, and 100-% seawater which was prepared from distilled water and C.P. chemicals according to the formula of Lyman and Fleming (1940). An additional .144 gram NaHCO<sub>3</sub>/liter was added to Lyman and Fleming's formula for a purpose apart from the present discussion. The salinity of the 100% seawater was 32.18 parts per thousand

The 346 ppm CO<sub>2</sub> concentration used in these experiments was orders of magnitude less than those in the studies with Chlorella. A Beckman Model 864 infrared analyzer operated at its most sensitive range, 0-500 ppm, provided the analysis for CO<sub>2</sub>. Because the proper use of this equipment required that the test gas be passed through at approximately 100 cc/min, and because the flow rate in our experiments was only 10 cc/min, it was not possible to monitor the CO<sub>2</sub> in the effluent on a continuous basis. The air was collected, therefore, in a 100-cc syringe opened at a rate which maintained constant (atmospheric) pressure within the tube; the syringe was then taken from the



apparatus and the air expelled through a small drying column into the analyzer. The equipment used is represented in Figure 11. The glass tube was 121.9 cm (4 feet) long and contained 85 ml of test water. Air bubbles were admitted through a glass tube, 2 mm ID, sealed into the throat of a ball joint and extending 2 mm above the opening in the joint. The ball joint was mated with the appropriate socket which was, in turn, fused to the bottom of the long glass tube. At the top of this tube was a plastic ell connected to a T-tube, the center leg of which extended into a reservoir of dilute acid. The connection from the other end of the T-tube to the glass syringe was made with a ball and socket joint. In taking the air sample, the plunger of the syringe was pulled open at the rate required to keep the meniscus in the dilute acid at its rest point. The time required to fill the syringe was measured to give the air flow rate. The four valves shown at the bottom of the diagram controlled the source and flow rate of air. Thermostated water was passed through the jacket surrounding the tube at a constant rate.

Prior to the start of an experiment the pressure regulators on each air cylinder were adjusted to 20 psi; a pressure of exactly 10 psi was maintained on the gauge shown in the figure by opening the valve leading to the proper air cylinder. Flow rate through the glass tube was maintained by the needle valve at the lower right portion of the diagram, and air was allowed to escape at a constant rate through the valve at the top right for CO<sub>2</sub> analysis. With this arrangement, there was no disturbance of the air/water equilibrium when the need arose for an analysis. To change from one CO<sub>2</sub> concentration to another required only the closing of one valve and the opening of another, which took less than eight seconds. Experiments were of two types: in one, the water was first equilibrated (i.e., nearly so) with 0 ppm CO<sub>2</sub> after which 346 ppm CO<sub>2</sub> air was bubbled through and the other was the converse, with initial aeration with 346 ppm CO<sub>2</sub> followed by 0 ppm CO<sub>2</sub>.

Some of the experiments included pH measurements which required that the connection at the top of the glass tube be modified to accommodate a Markson electrode which was connected with an Orion Model 801 meter.

The formula used to calculate the absorption (or release) rate of CO<sub>2</sub> is shown below:

$$\text{cc CO}_2/\text{min} = \frac{\Delta \text{CO}_2 \text{ (ppm)} \times \text{air flow rate (cc/min)}}{10^6}$$

The principal results of these equilibration studies are represented in the next four figures. Figure 12 is a plot of pH and the volume of CO<sub>2</sub> released per minute from a 25% seawater solution (salinity = 8.24 ppt) which had been initially at equilibrium with 346 ppm CO<sub>2</sub> but then aerated with 0 ppm

CO<sub>2</sub>. The starting pH was 8.01 and in this case the final was 9.00, but the upper pH limit is a function of aeration time and not nearly so well defined as that when equilibrium with air containing CO<sub>2</sub> is established. Figure 13 represents data from the converse experiment in which 25% seawater, initially almost at equilibrium with 0 ppm CO<sub>2</sub>, was aerated with 346 ppm CO<sub>2</sub>; CO<sub>2</sub> uptake was complete in about five hours and the pH ended up exactly at the starting point for the previous experiment.

To demonstrate conveniently the relative rates of CO<sub>2</sub> absorption as a function of salinity requires that an arbitrarily selected rate of absorption be used as the starting point of the plot, otherwise the crisscrossing of lines with varied slopes becomes confusing. In Figure 14, the zero time point corresponds to an uptake rate of  $2.56 \times 10^{-3}$  cc/min; beyond that point, only 200 minutes were required for equilibrium to be established with 25% seawater but more than 800 minutes were required with the 100% seawater.

With the technique described here it was easy to measure the uptake or release per minute of CO<sub>2</sub> amounting to only 0.0026% of the total dissolved CO<sub>2</sub>. These figures refer to undiluted seawater and an air flow rate of 10 cc/min.

Figures 12 and 13 demonstrate the relationships between pH and release, or absorption, of CO<sub>2</sub> by a 25% seawater solution which has either been saturated or stripped of CO<sub>2</sub>. Time zero in these plots marks the changeover from one air cylinder to another. An appreciation of the relative sluggishness of the pH response compared to that of gas exchange is demonstrated by a different use of the data used in Figure 13. Figure 15 contains data on pH and CO<sub>2</sub> prior to, and after, the change from one air stream to another and the plot of CO<sub>2</sub>/minute includes both negative and positive values. Prior to time zero there was a very slow release of CO<sub>2</sub> though the stripping operation had been proceeding overnight, and during this time the pH rose at a rate of about .001 unit in 5 minutes. With the change at time zero, from 0 ppm to 346 ppm CO<sub>2</sub>, the pH continued to rise for several minutes before starting to drop slowly. Concurrent with the slow pH change was an enormous change in the composition of the air exiting from the system. At this point the minimum pH change corresponded with a maximum change in CO<sub>2</sub> absorption.

The complexity of the equilibration of CO<sub>2</sub> with waters of various salinities is demonstrated in the figures shown. Rates of change of CO<sub>2</sub> uptake or release depend upon both pH and salinity. To summarize the research on limitations of the gas exchange approach, with CO<sub>2</sub> in waters of various salinities, the salinity should probably be below 13 parts per thousand otherwise the gas exchange rate is too slow for meaningful measurements.

#### IV. DISCUSSION

The thrust of this report is to emphasize the speed with which changes take place in the  $O_2$  or  $CO_2$  content of air passing through cultures of microorganisms, compared to changes in biomass. Three factors prompted an awareness of this phenomenon in the research at NRL:

- a. The Chlorella used in the algal studies had an extraordinarily high  $O_2$  production rate. The six-lamp unit could produce over 700 cc  $O_2$ /liter/hr and another unit featuring an illuminated area only 1 cm deep produced more than double that amount.
- b. High air flow rates could be used to advantage in such cultures which, in turn, resulted in a fast equilibration between the air and the liquid.
- c. The Chlorella cultures had a low salinity which also was responsible for fast air/liquid equilibration.

In this report the emphasis has been on the rapidity with which changes in  $O_2$  production of an algal culture can be detected following the imposition of physical or chemical changes on the system. Pressure,  $CO_2$  input rate, light intensity, and the presence of a toxic alloy were shown to induce practically immediate responses in terms of the  $O_2$  production rate, whereas nutrient changes (either by the addition of extra urea, or by the substitution of a particularly concentrated culture medium) resulted in more gradual changes in  $O_2$  production. Neither packed cell volume nor cell number is a sensitive measure of the productivity of an algal culture, largely because the  $O_2$  production rate is a function of illuminated area of the culture once enough cells are present to make the culture light-limited. Therefore, biomass changes of an algal culture are often unsatisfactory criteria of its performance; in contrast, the changes in  $O_2$  production rate can be remarkably fast and easily detected.

The instances cited have concerned either  $O_2$  or  $CO_2$  as components of an air stream but the concept is not limited to these two gases. The same consideration would apply to any gas for which a sensor is available.

##### Reproducibility

The most compelling argument for the reliability of an algal culture as a producer of  $O_2$  rests on the results obtained with the six-lamp unit over a five-day period, when the total variation in results could be accounted for by misreading the  $O_2$  analyzer by only 0.01%. Under controlled conditions of light intensity,  $CO_2$  aeration rates, etc., algal cultures will behave

predictably. The responsibility of the researcher is to ensure that the effluent air being analyzed is representative of air at equilibrium with the system. The main concern here is air flow rate; if, for some reason, the air flow rate were reduced momentarily the result would be a change in the air composition. It is necessary, therefore, to keep the flow rate constant and to allow enough time for the effluent to sweep out the volume contained in the drying column and the analyzer before a reading is taken.

The reproducibility of gas exchange studies was demonstrated also in the studies of equilibration of CO<sub>2</sub> with 25% seawater. Three experiments were performed in which the seawater was equilibrated with 346 ppm CO<sub>2</sub> after an initial aeration with 0 ppm CO<sub>2</sub>. The initial pH's were different but the results obtained, relating CO<sub>2</sub> uptake vs. time, gave super-imposable plots when adjusted to a fixed uptake value.

Temperature can be an important factor. In the studies concerning equilibration with dilute seawater, it was necessary to determine the point when equilibrium had been reached, i.e., when the effluent air had the same CO<sub>2</sub> content as the input. Instances in which the equilibrium was obviously delayed could usually be traced to minor changes in temperature. An elaborate system of temperature control, which limited the variation to less than 0.06°C during the course of an experiment, solved the problem. The problem was less acute with the large algal culture unit, perhaps because at its temperature (39°C) the rate of change of CO<sub>2</sub> solubility per degree centigrade is less than half that at 22°C.

Bubble size can be an important factor. It was not a focal point in these studies but an insight into its importance was obtained when one bubbler was substituted temporarily for another in the CO<sub>2</sub> equilibration studies. The difference in the diameters of the two tubes was not apparent to the eye but the gas exchange characteristics were greatly different.

#### Additional Factors to be Considered

There may be a misconception that the O<sub>2</sub> production of an algal culture cannot be constant and that it must increase as growth proceeds. That happens to be the case with extremely dilute cultures when all the cells are subjected to the same light intensity at all times. This was not the situation with the rather dense cultures (greater than 1% packed cell volume) used in the Chlorella studies where only a thin layer of cells closest to the light received the full intensity; as mentioned earlier, in such "light limiting" conditions the productivity of the culture is a function of the area of illumination and the O<sub>2</sub> production is constant over quite a wide range of cell concentrations.

To this point, mention has been made of  $\text{CO}_2$  and  $\text{O}_2$  measurements as single entities but in many instances the combination of the two can provide additional information. The assimilatory quotient for algae ( $\text{O}_2/\text{CO}_2$ ) varies according to the nitrogen source being utilized by the algae. This is well known and will not be explored further at this point. What, perhaps, is not so well known is the variation of the assimilatory quotient with the condition of the organism. The Chlorella cultures, with urea as the nitrogen source, had an AQ of 1.06 when all conditions were favorable for growth. But a culture which had been refrigerated over a weekend would have an AQ of 0.9 or even less for at least several hours in the mass culture apparatus. It is useful, therefore, to know what the expected AQ is in order to make a judgment about the well being of the culture at any time.

The better known respiratory quotient ( $\text{CO}_2$  produced/ $\text{O}_2$  absorbed) has been a useful diagnostic in medical research on humans and animals, though there are instances in which its particular relevance have been overlooked. A case in point [work not performed at NRL, obviously] concerns the effect of certain aflatoxins occurring in the feed given to chickens; for a period of several weeks' exposure to these compounds the chickens have no visible defects, but with the routine handling associated with the killing of the chickens and removal of their feathers, the blood vessels hemorrhage, resulting in a considerably lower sale price of the meat. Clinical investigations have shown that an effect of the aflatoxins is to decrease the ability of the chickens to absorb fat from their diet, and fats in the feed pass through the animal into the feces without digestion. The difference between the respiratory quotient of a healthy chicken, and one exposed to aflatoxins, should be detectable and could serve as a warning that an abnormal condition existed.

#### Alloy Toxicity

The most intriguing research included in this report concerns the inhibition of  $\text{O}_2$  production by Chlorella when exposed to 3.8 mm diameter rods made of certain alloys. Dense cultures (approximately 1.5% packed cell volume) were used in this study and two questions come to mind when contemplating such remarkable results: 1) why is the  $\text{O}_2$  production changed so abruptly, and to a new constant level, rather than a slow decline which one might expect with the imposition of a dilute toxic agent?; 2) how can such a dense culture be affected by what must be minor concentrations of extractable metallic species?

The answers to these questions are not known. In that particular study, an attempt was made to duplicate the decline in  $\text{O}_2$  production by introducing varying concentrations of metal ions of the alloy components ( $\text{Cr}^{3+}$ ,  $\text{Cr}^{6+}$ , etc.) but

the results were not definitive. Also, the concentrations required to produce such inhibitions were too high to be regarded as realistic values. The results obtained with one rod would support the notion that leakage of some particularly toxic component from a grain boundary was the source of the inhibition. Perhaps an exotic species vastly different from the single ion components tested could be responsible for the inhibitions noted.

In all deliberations on this subject, the focus has been on toxicity. It may be more accurate to regard changes in  $O_2$  productivity as the result of a change in synchrony of the cells. Sorokin (1963) showed (Figure 16) the changing rates of photosynthetic gas exchange for ten-minute intervals in cells that were synchronized. The new (0-hr) cells showed a progressive rate of gas exchange while the older (8-hr) cells showed a decline. Therefore, a drastic change in synchrony, in a population which is totally diverse in terms of cell age, could result in a change of  $O_2$  production rate. Once again, however, is the problem that an explanation be given for the release of sufficient material from the metal alloy in question to cause a change in synchrony of the cells.

For the purpose of this discussion it is not necessary to resolve the dilemma although it would certainly be desirable. The essential point is that an algal culture can respond to a specific metal alloy specimen in a very short time which presents an opportunity for exploitation. A likely possibility concerns the study of marine corrosion, and the connection between corrosion and algal susceptibility to toxicants is based on the following line of thought: a) inhibition of  $O_2$  production by an alloy is a surface phenomenon; b) leaching of constituents from a grain boundary in the surface is a plausible explanation for the effect of an alloy on an algal culture; c) resistance to corrosion by an alloy may be reduced by the loss of the same constituents. If the premise is valid, then an initial screening test of alloy specimens for toxicity (particularly the stainless steels) might be correlated with corrosion susceptibility in a field test. This is admittedly a simplistic explanation for what must be a difficult field of study, but metallurgists seeking an explanation for the occasional, unusual, corrosion problem might consider this possibility.

Convincing arguments for the greater use of gas exchange studies could be generated by researchers in diverse fields who are aware of the existing problems in those fields. The instances of applications cited here concern algae for the most part, but also include animal experimentation and metallurgical research. Additional work being done at NRL, but not included in this report, concerns the  $CO_2$  production rate of baker's yeast as the central point of an assay for the detection of certain toxins. Preliminary results are extremely promising in that, in certain instances, the effect of only 2 micrograms

of toxin is apparent in only 12 minutes.\* All of these methods have in common the need for control over air flow rate and temperature and a CO<sub>2</sub> or O<sub>2</sub> analyzer. The costs for equipment are modest compared to so much of the experimentation which is found in most research laboratories. The essence of the gas exchange approach is that each measurement is a rate measurement -- those who have an appreciation of this fact should be interested in further studies.

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Table 1 — Burk's medium

Major Constituents	Amount (grams/liter)
MgSO <sub>4</sub> 7 H <sub>2</sub> O	5.0
KH <sub>2</sub> PO <sub>4</sub>	2.5
NaCl	2.0
Urea	0.4

Micronutrients	Conc. Source Compd. (mg/liter)	Conc. Trace Element (ppm)	M.E.Q. Element (liter)
Fe — EDTA	4	1 Fe	$5.36 \times 10^{-2}$
CaCl <sub>2</sub>	22	8 Ca	.8
H <sub>3</sub> BO <sub>3</sub>	5.7	1 B	.6
MnCl <sub>2</sub> 4 H <sub>2</sub> O	3.6	1 Mn	$3.64 \times 10^{-2}$
ZnSO <sub>4</sub>	0.44	0.18 Zn	$5.5 \times 10^{-2}$
CuSO <sub>4</sub> 5 H <sub>2</sub> O	0.158	0.04 Cu	$1.25 \times 10^{-2}$
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> 5 H <sub>2</sub> O	0.035	0.019 Mo	$1.19 \times 10^{-2}$
NaVO <sub>3</sub>	2	0.84 V	$8.24 \times 10^{-2}$

Table 2 — Summary of week-long performance of six-lamp unit

Light Intensity: 23,000 Foot-Candles  
 Electrical Power: 7040 Watts  
 Dilution Rate: 15.6% per hour  
 Gas Flow Rate: 4000 cc/min, containing  
 1.9% CO<sub>2</sub>

Day	Suspension Density Percent Packed Cell Volume	Oxygen Production		Dilution Rate (ml/hr for 24 hr)
		Volume (cc/hr)	Standard Deviation (cc/hr)	
Monday	1.27 - 1.37	3432	145	Not measured
Tuesday	1.44 - 1.50	3555	30	964
Wednesday	1.43 - 1.47	3606	29	969
Thursday	1.41 - 1.44	3584	25	966
Friday	1.41 - 1.43	3638	24	950

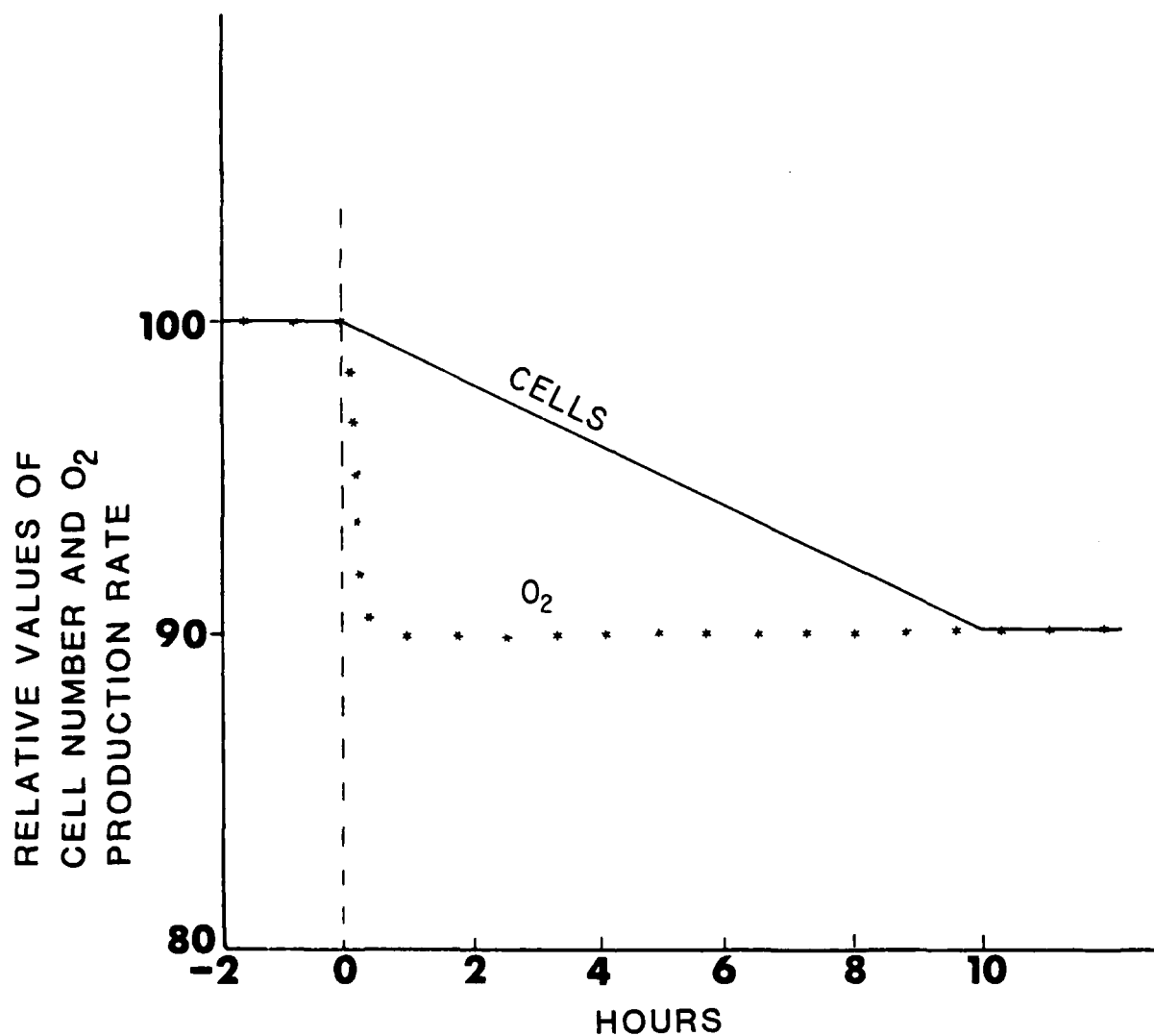


Fig. 1 — This is a theoretical plot of the cell number and the O<sub>2</sub> production rate, vs time, of an algal culture being diluted with fresh medium at 10% hr<sup>-1</sup>. At time zero the growth rate was reduced 10% from the original.

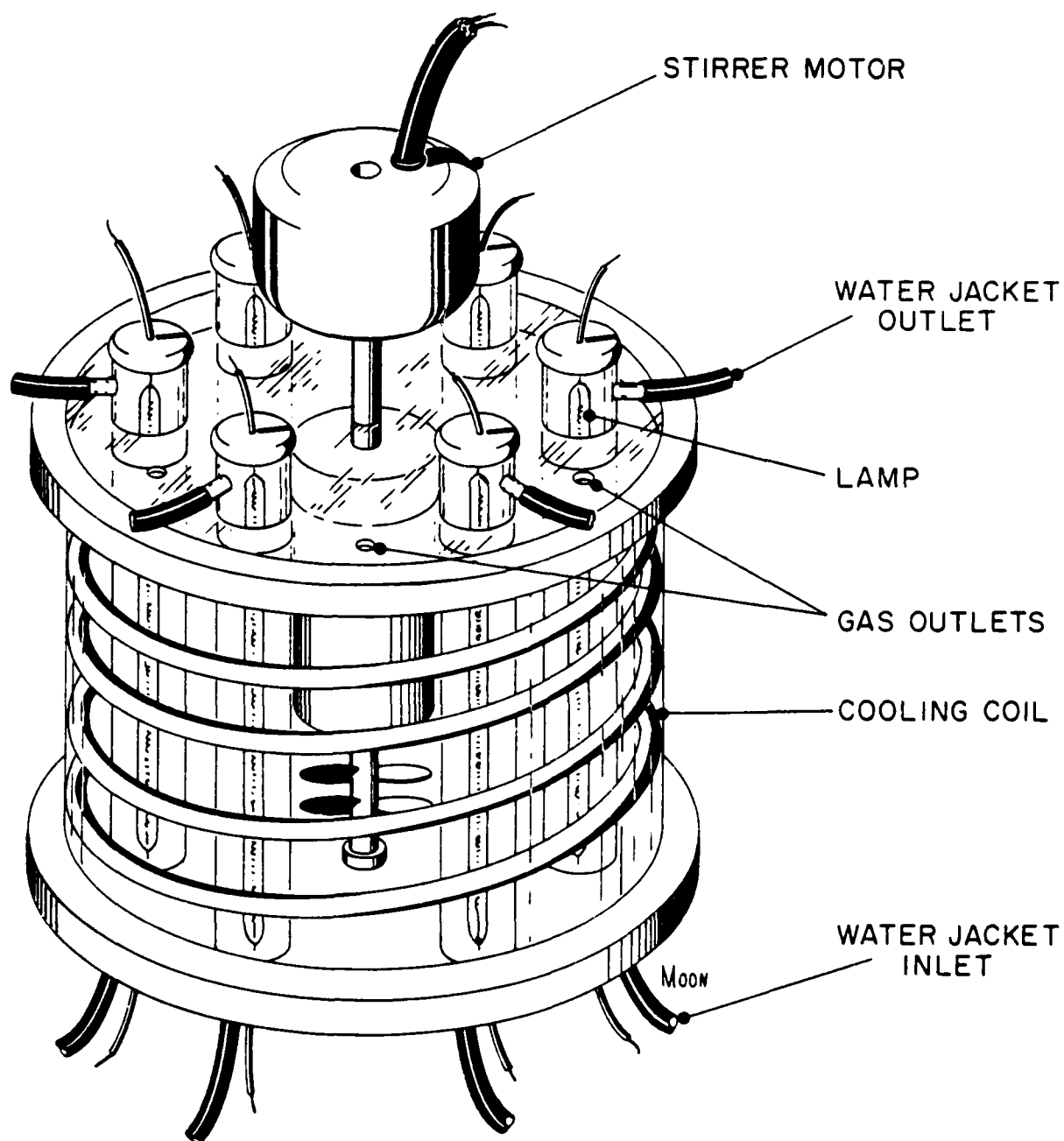


Fig. 2 — Schematic representation of a mass culture unit containing six Quartzline high intensity lamps

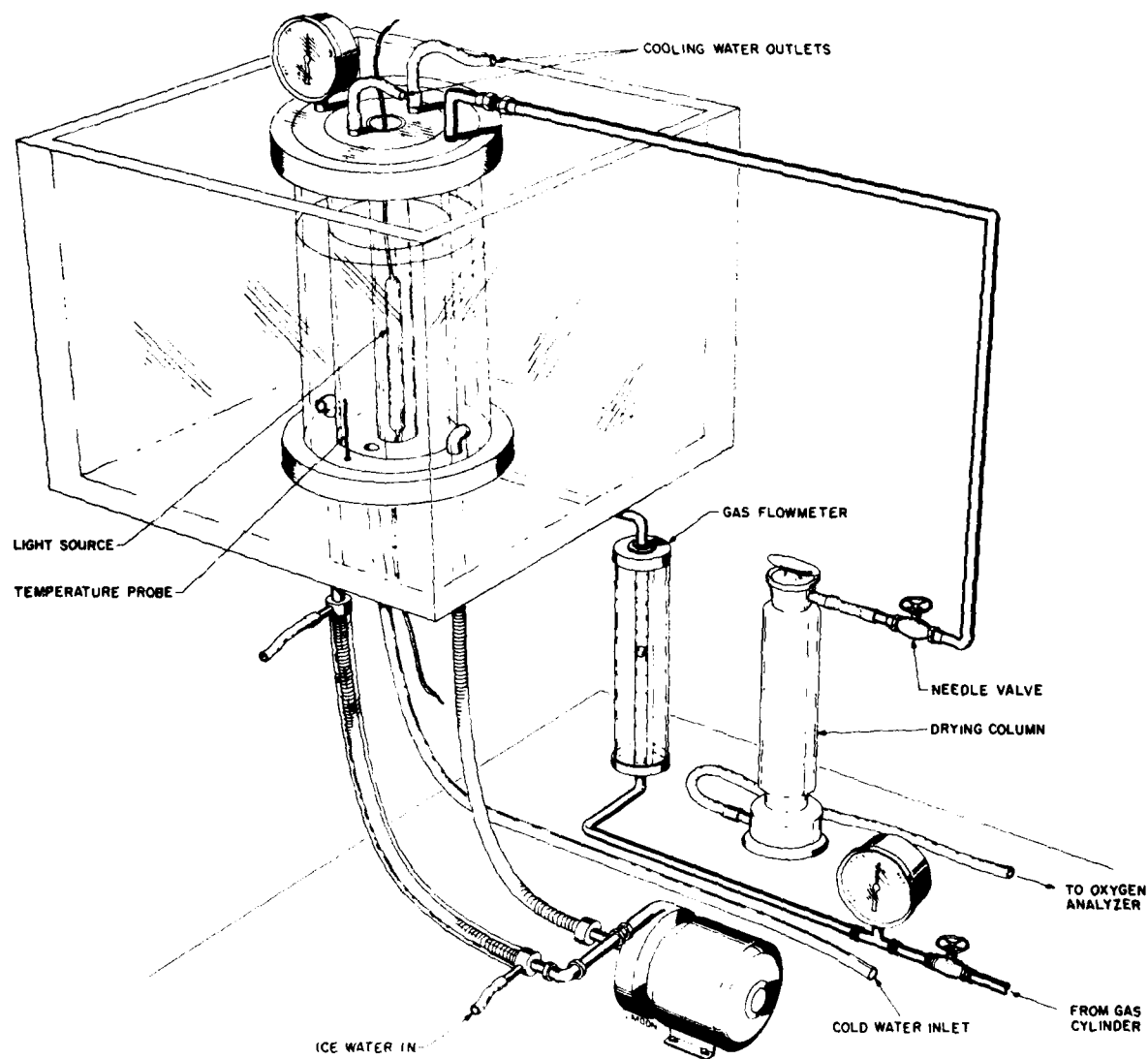
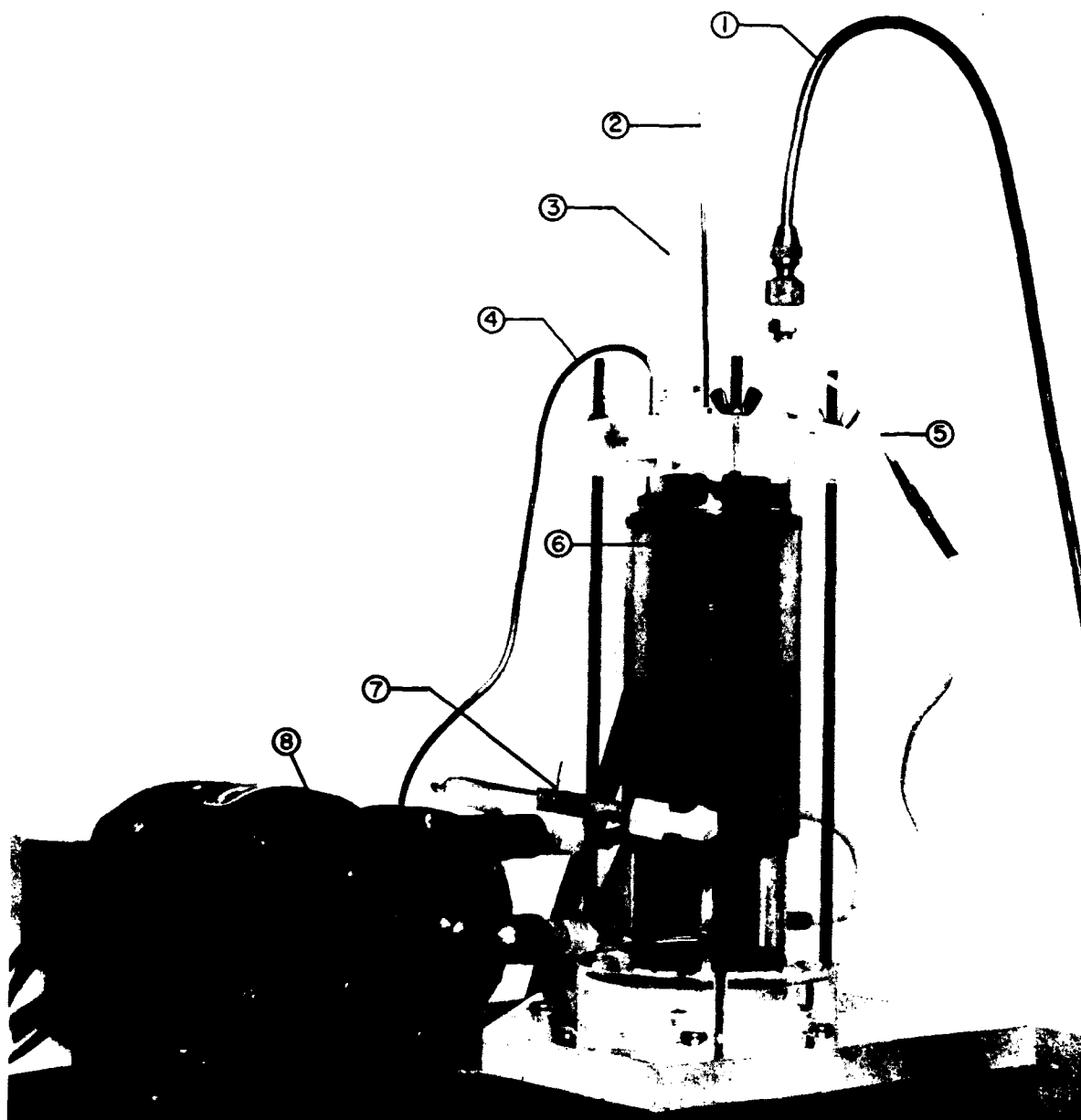


Fig. 3 — Diagram of a culture apparatus, made from glass pipe and end plates of Plexiglas, which was used for the study of pressure effects on *Chlorella*. An inner annular space contained water to dissipate the heat released by the Quartzline lamp extending through the unit. The algal culture, in the outer annulus, was circulated by a centrifugal pump underneath this unit.



- |                             |                                      |
|-----------------------------|--------------------------------------|
| ① AIR INLET TUBE            | ⑤ POWER CABLE FOR LIGHT SOURCE       |
| ② METAL SAMPLE ROD          | ⑥ OUTER JACKET OF ALGAE CULTURE UNIT |
| ③ COOLING JACKET OUTLET     | ⑦ COOLING JACKET INLET               |
| ④ TEMPERATURE SENSING PROBE | ⑧ ALGAE CIRCULATING PUMP             |

Fig. 4 — Algal culture unit used in toxicity studies. A light source extended vertically through the unit, surrounded by annular spaces containing cooling water and the algal culture; circulation of the culture was provided by the centrifugal pump. Additional cooling of the culture was provided by cold water, circulating through an outer jacket, on demand from a thermoregulator probe. A 1/8" diameter rod is shown extended above the unit; it is held in place by an O-ring seal.

## EFFECT OF LIGHT INTENSITY ON O<sub>2</sub> PRODUCTION

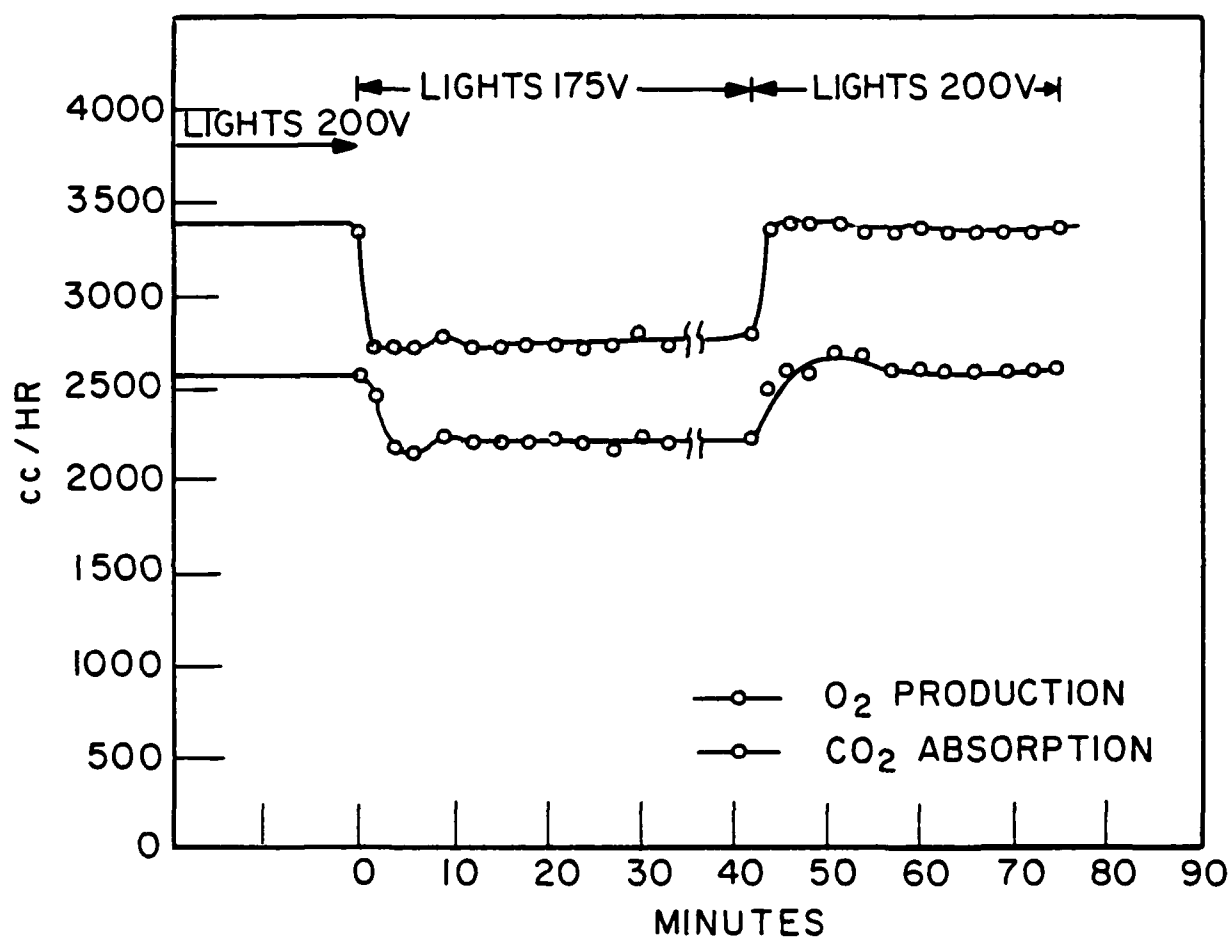


Fig. 5 — A plot of the O<sub>2</sub> production and CO<sub>2</sub> absorption rates of mass culture of *Chlorella* in response to light intensity changes.

# EFFECT OF CO<sub>2</sub> SUPPLY RATE ON GAS EXCHANGE

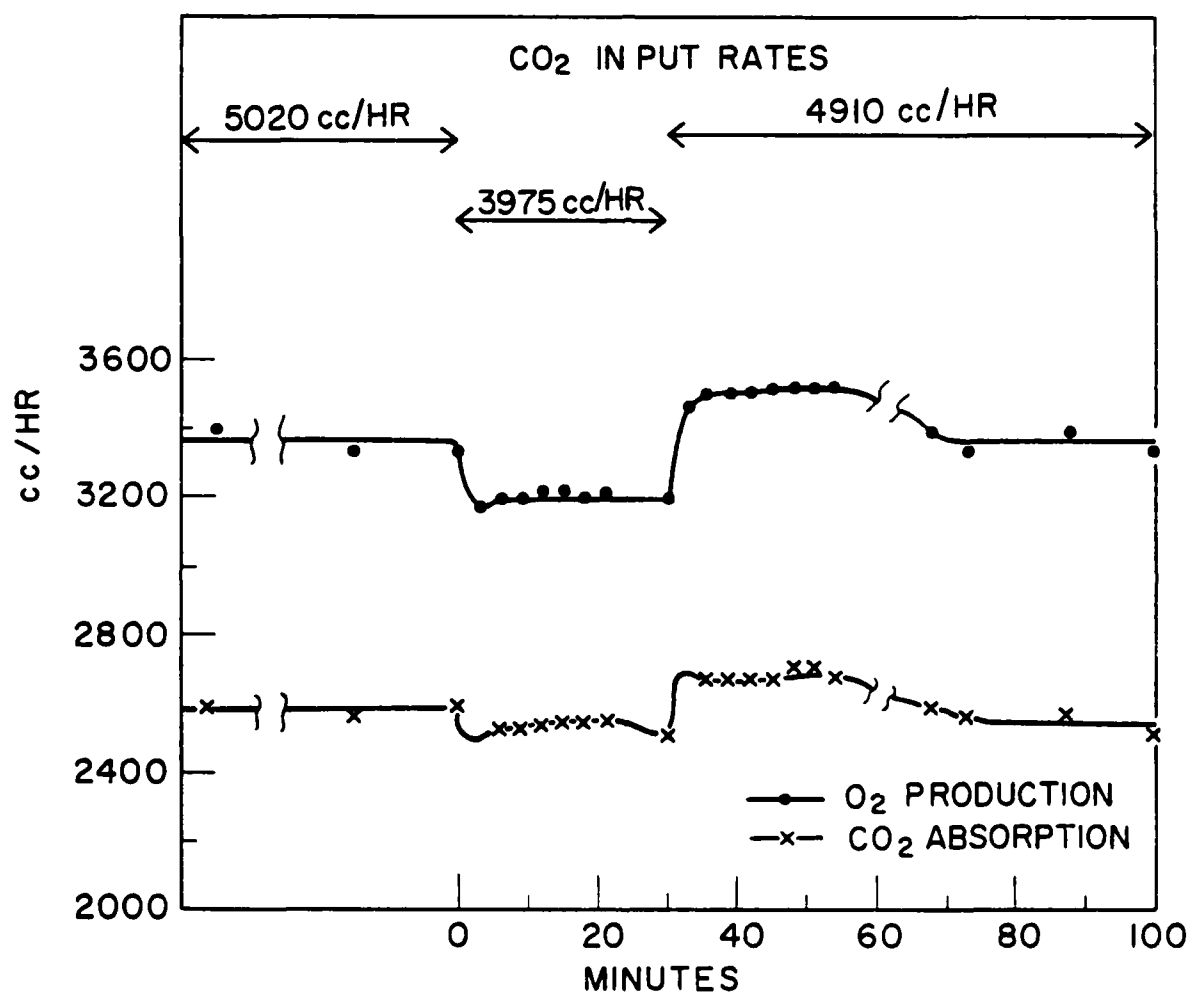


Fig. 6 — A plot showing the relationship between O<sub>2</sub> production, and CO<sub>2</sub> uptake, of a mass culture algae with changes in the CO<sub>2</sub> input rate.



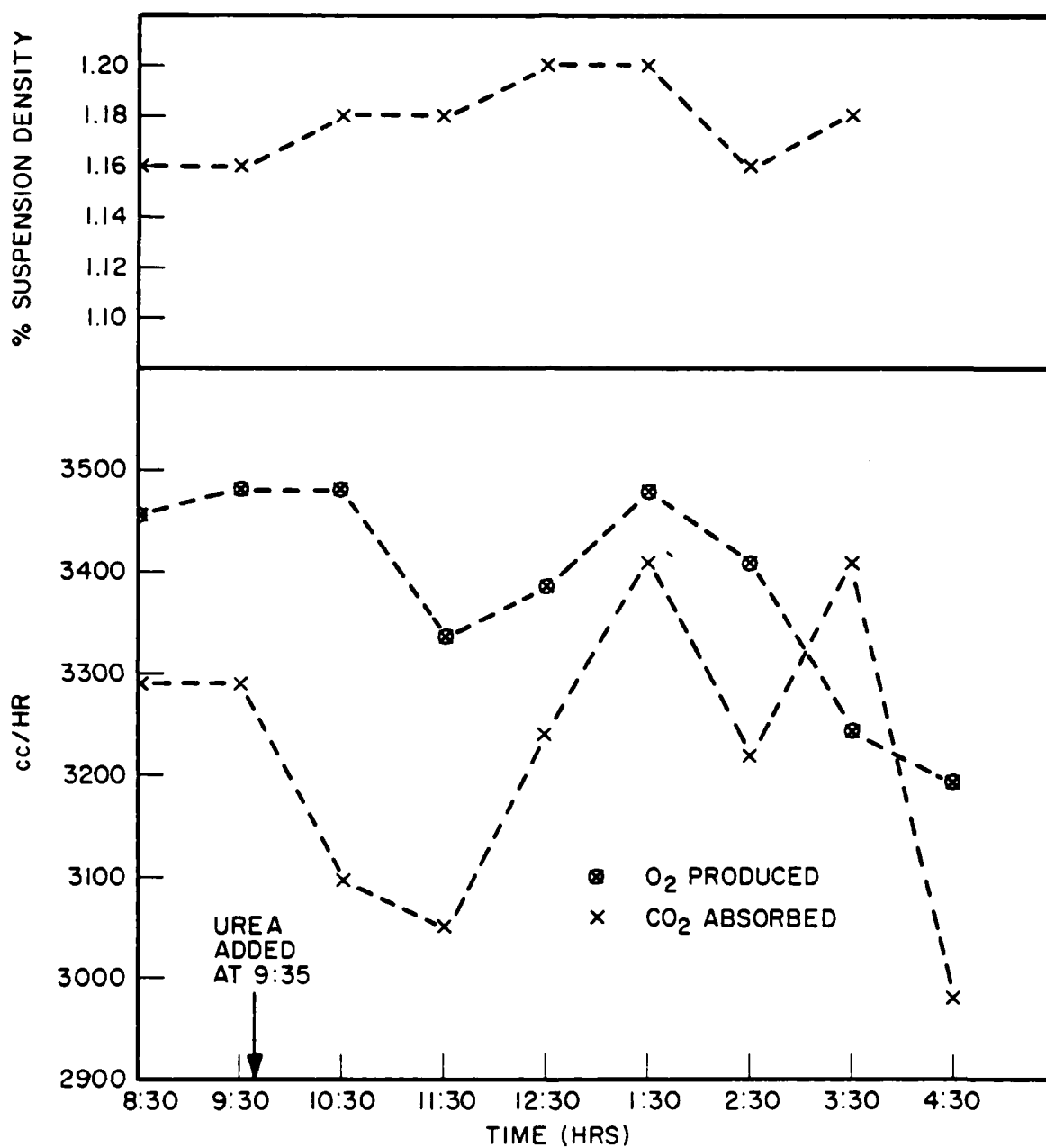


Fig. 7 — The effect of doubling the urea concentration of *Chlorella* which was being diluted at a constant rate of  $15\% \text{ hr}^{-1}$  with regular culture medium. Note the slight changes in suspension density in comparison with the large excursions in CO<sub>2</sub> absorption and O<sub>2</sub> production.

## ADAPTATION OF CELLS TO ENRICHED MEDIUM

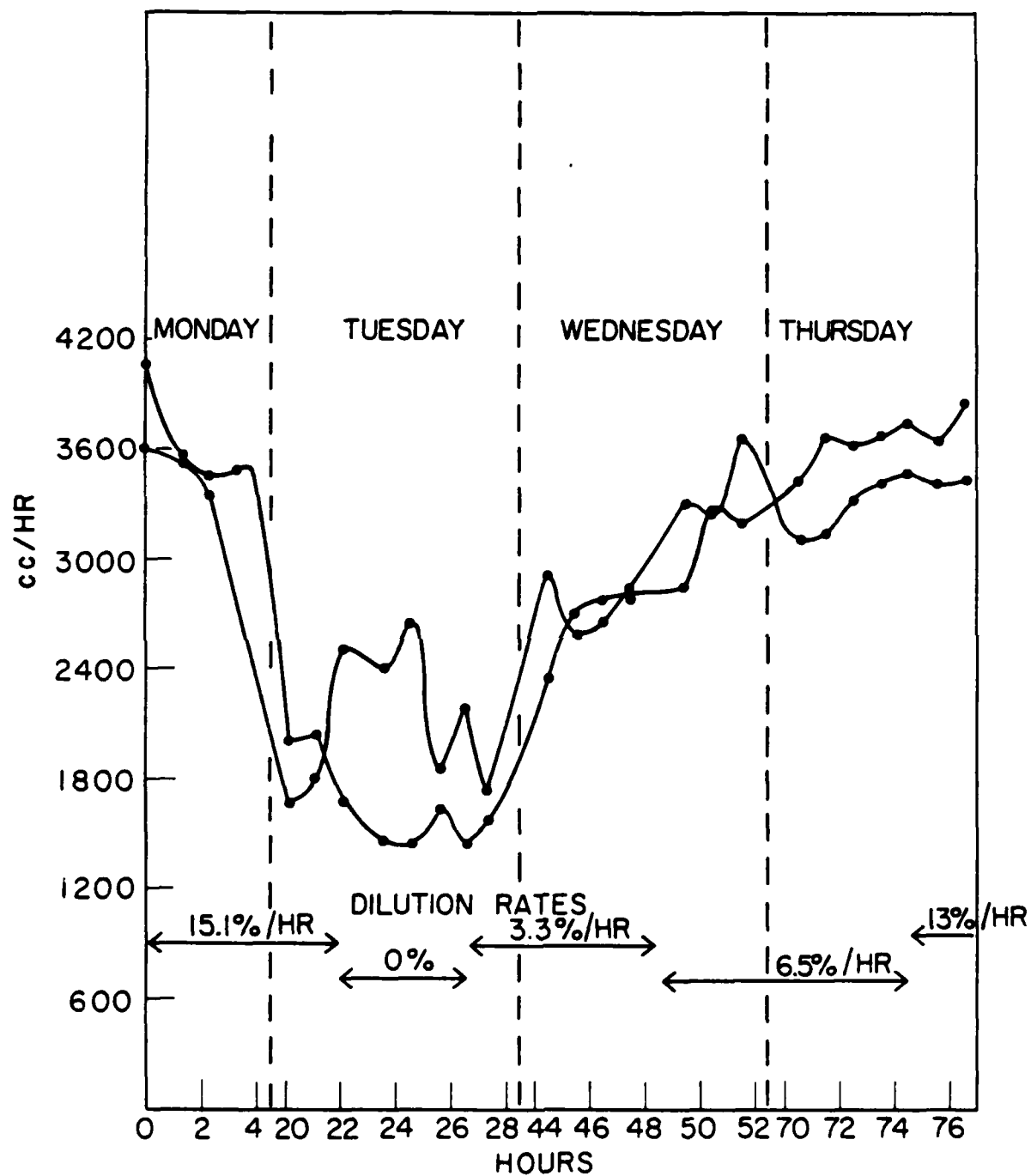


Fig. 8 — Variations in  $O_2$  production and  $CO_2$  uptake of mass culture of *Chlorella* following the substitution of a highly concentrated medium for the one normally used.

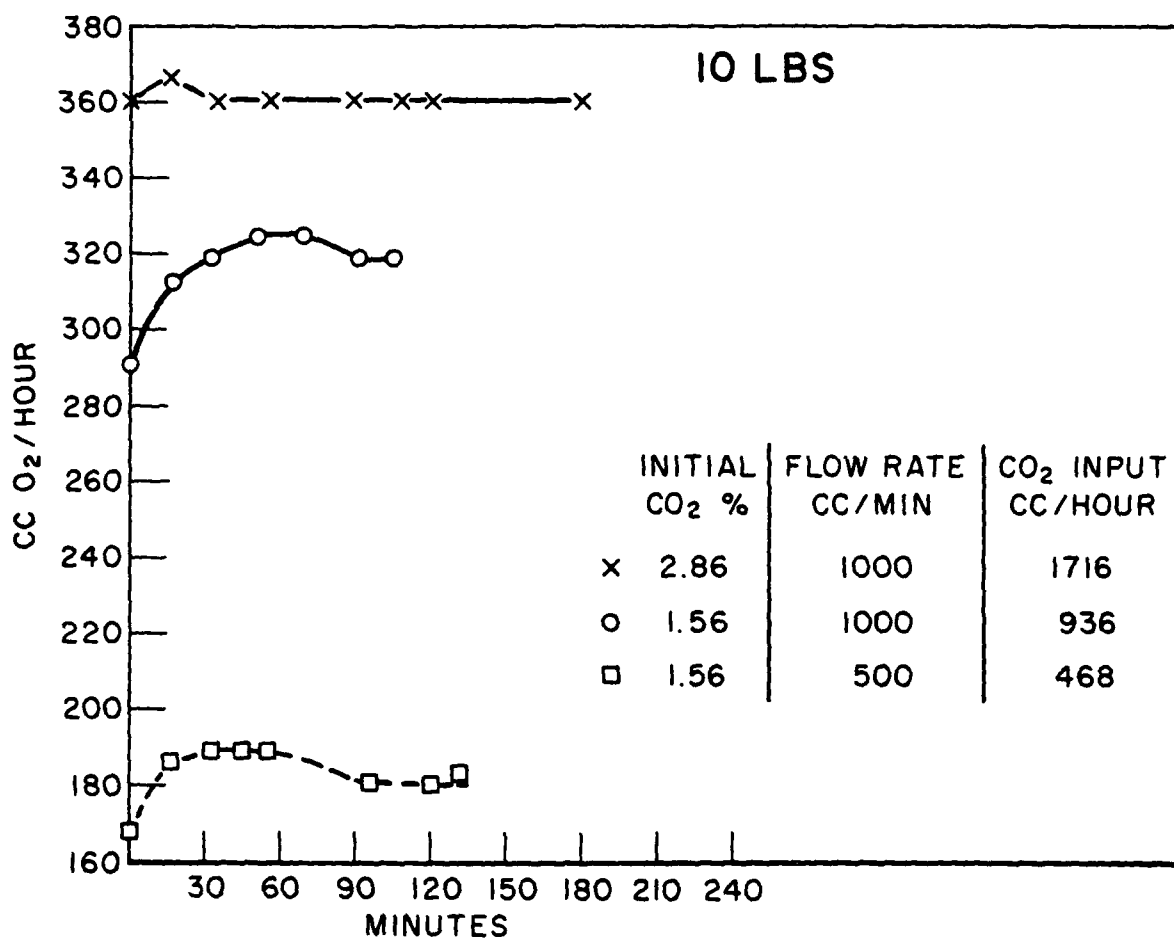


Fig. 9 — Plots showing the effects of various pressures, at three input rates, on O<sub>2</sub> production by *Chlorella*.

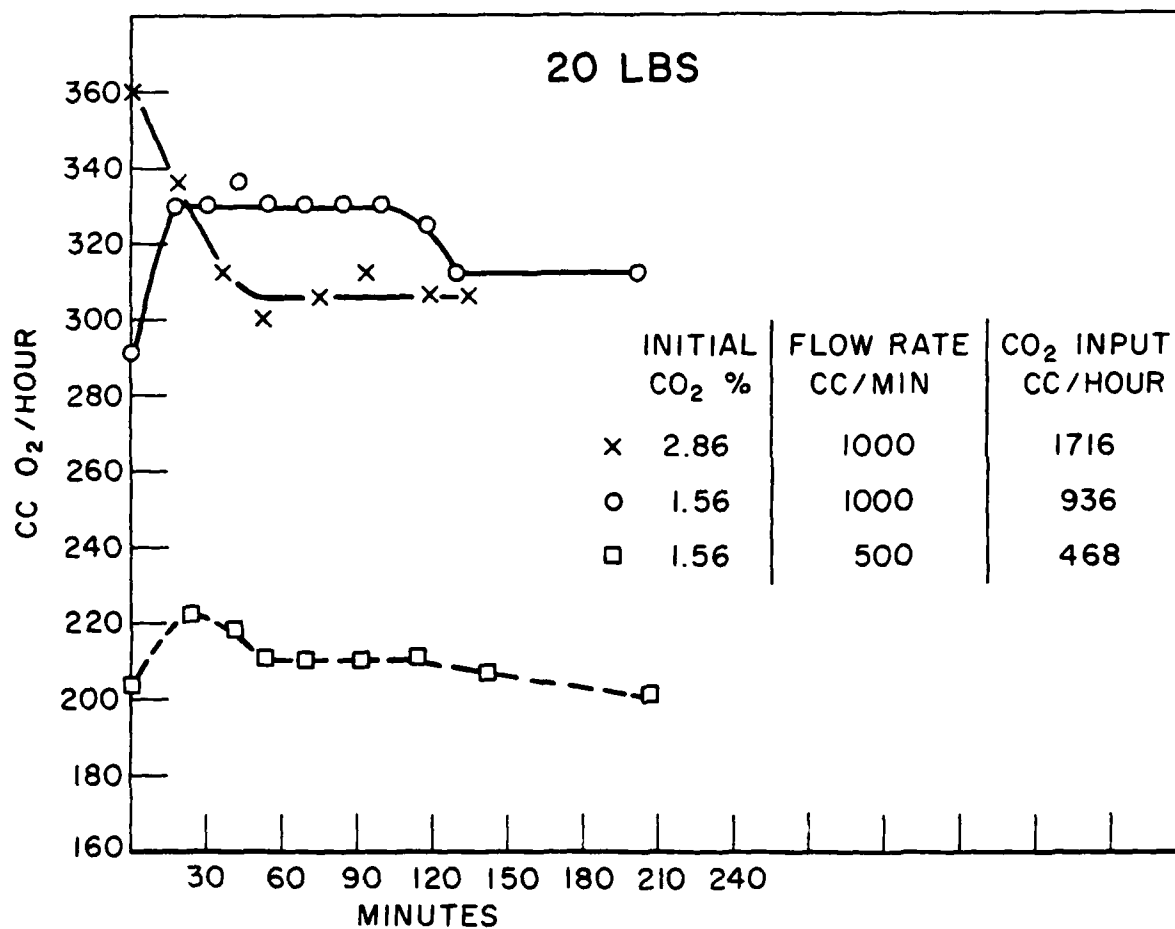


Fig. 9 (Cont'd) — Plots showing the effects of various pressures, at three input rates, on O<sub>2</sub> production by *Chlorella*.

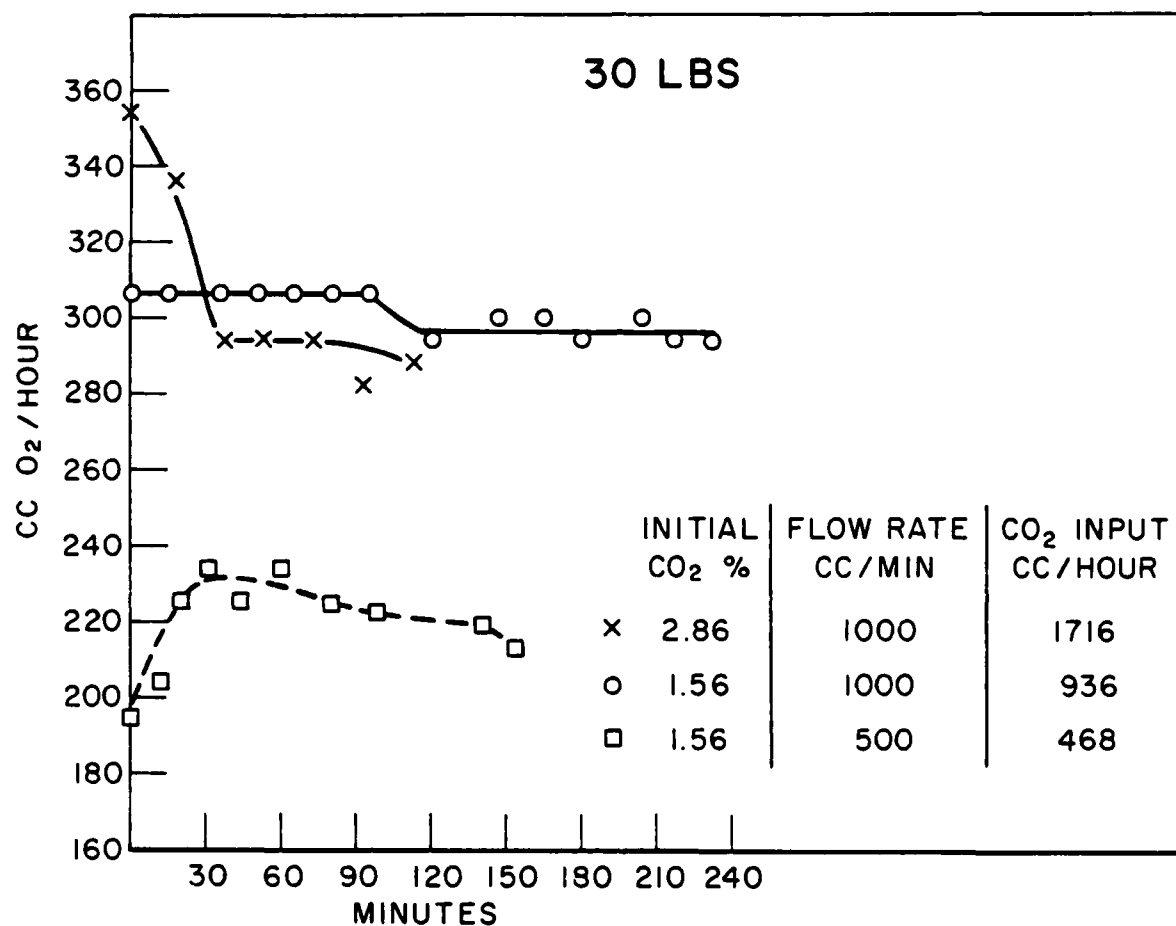


Fig. 9 (Cont'd) — Plots showing the effects of various pressures, at three input rates, on O<sub>2</sub> production by *Chlorella*.

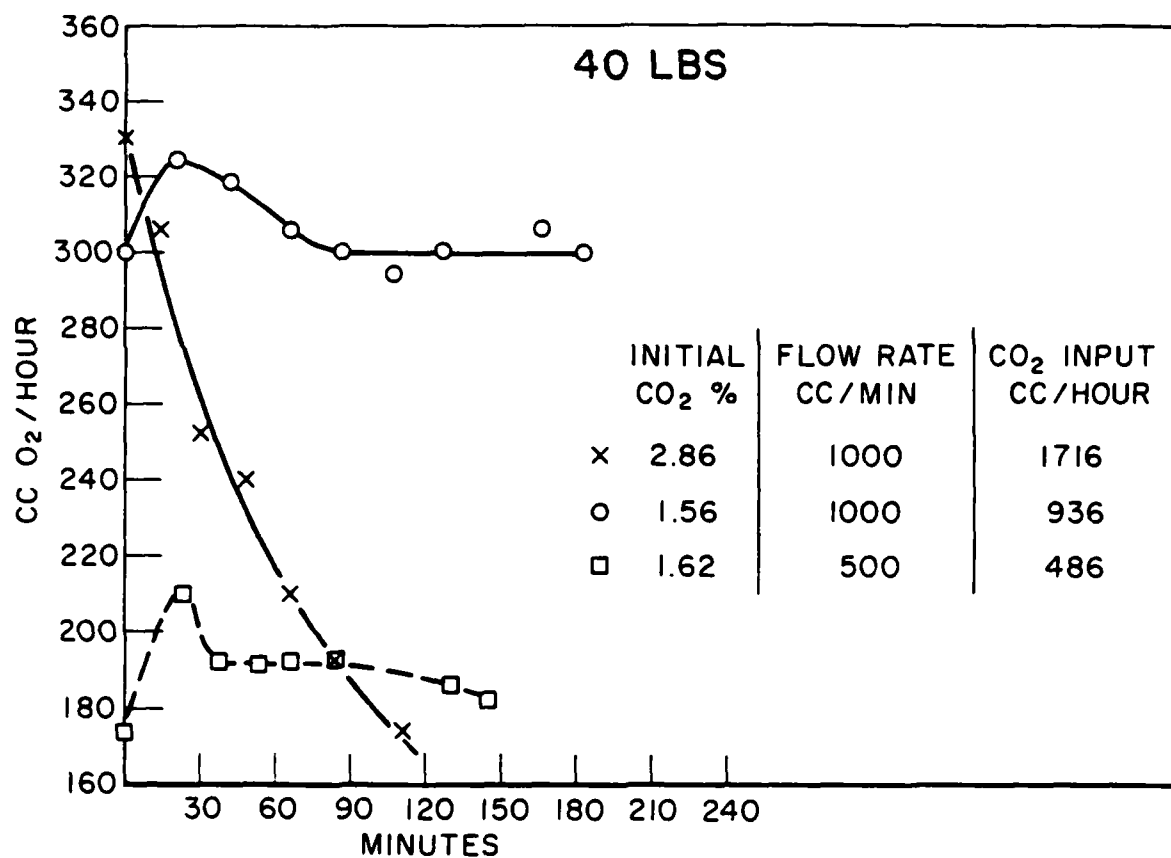


Fig. 9 (Cont'd) — Plots showing the effects of various pressures, at three input rates, on O<sub>2</sub> production by *Chlorella*.

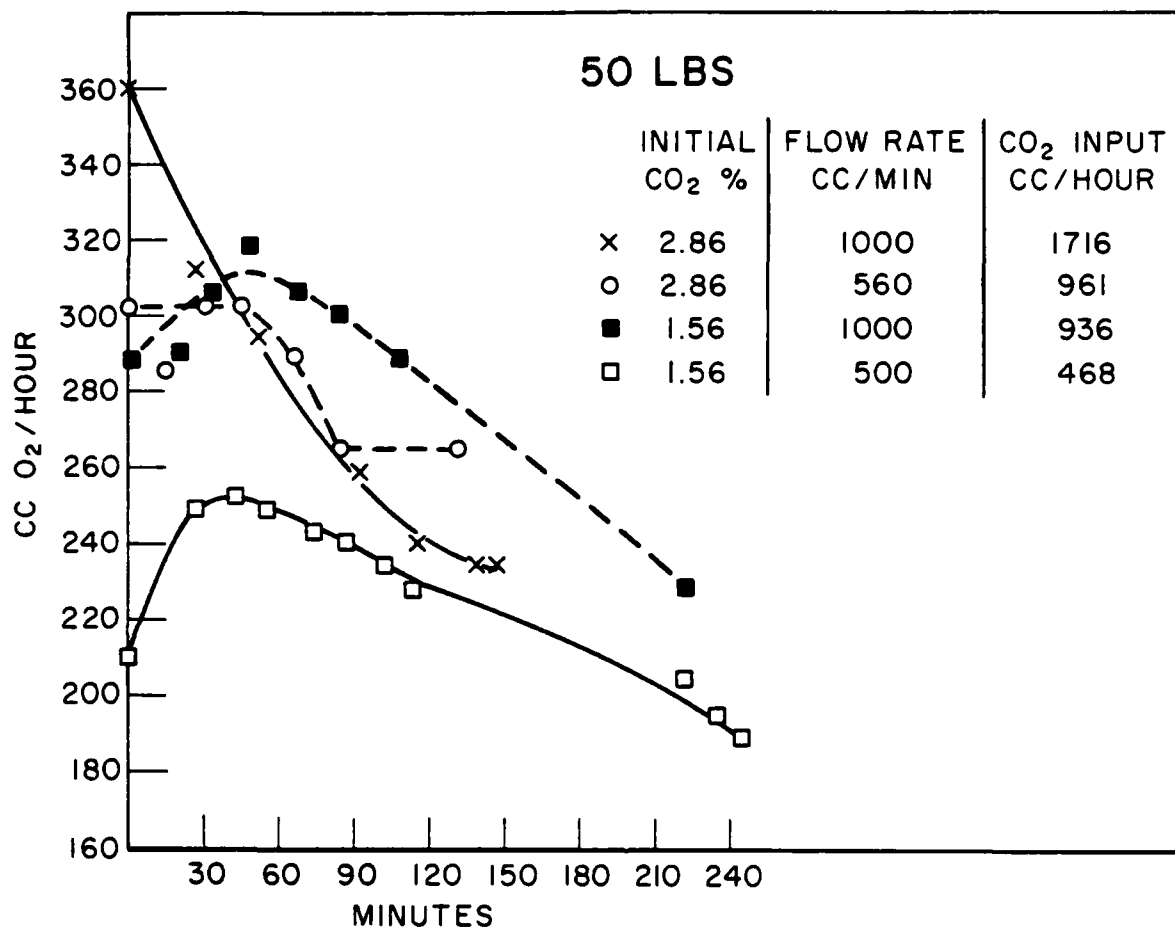


Fig. 9 (Cont'd) — Plots showing the effects of various pressures, at three input rates, on O<sub>2</sub> production by *Chlorella*.

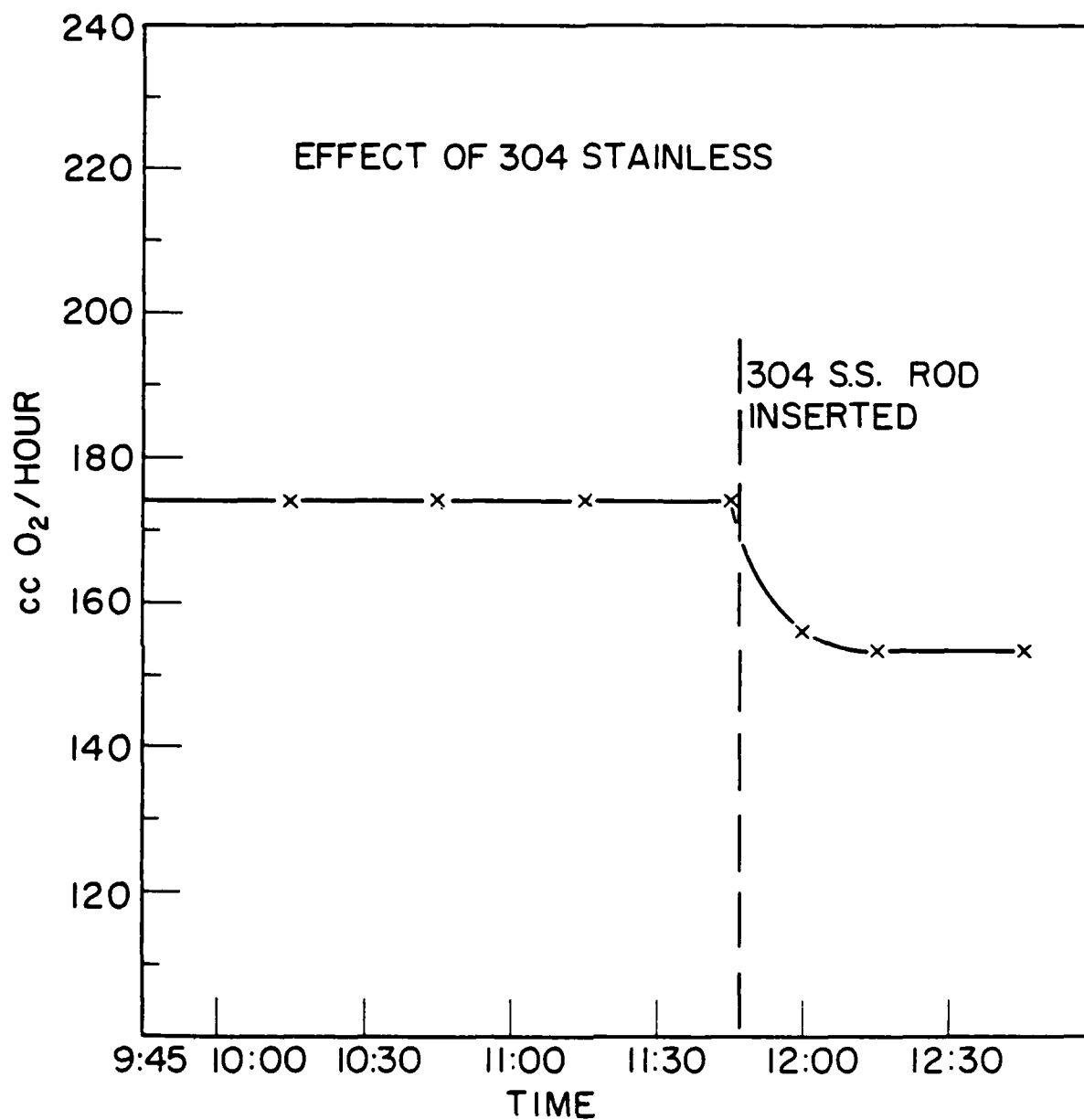


Fig. 10 — Oxygen production of an algal culture before and after the insertion of a 1/8" diameter rod of #304 stainless steel into the culture. In order to provide an expanded scale, the plot on the vertical axis is not extended to zero.



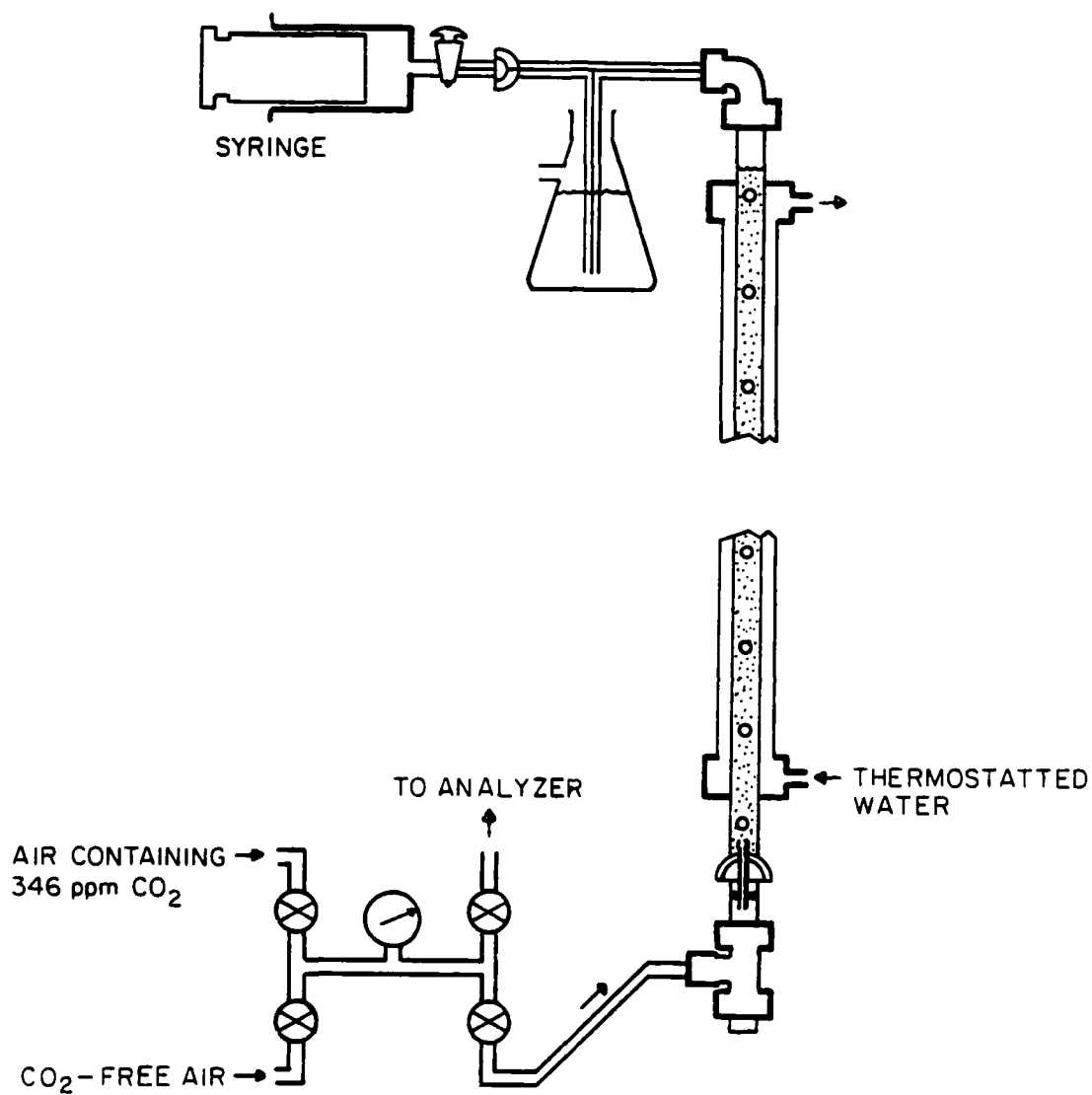


Fig. 11 — Schematic diagram of an apparatus used to study equilibrium rates of  $\text{CO}_2$ /air mixtures with seawater of various salinities.

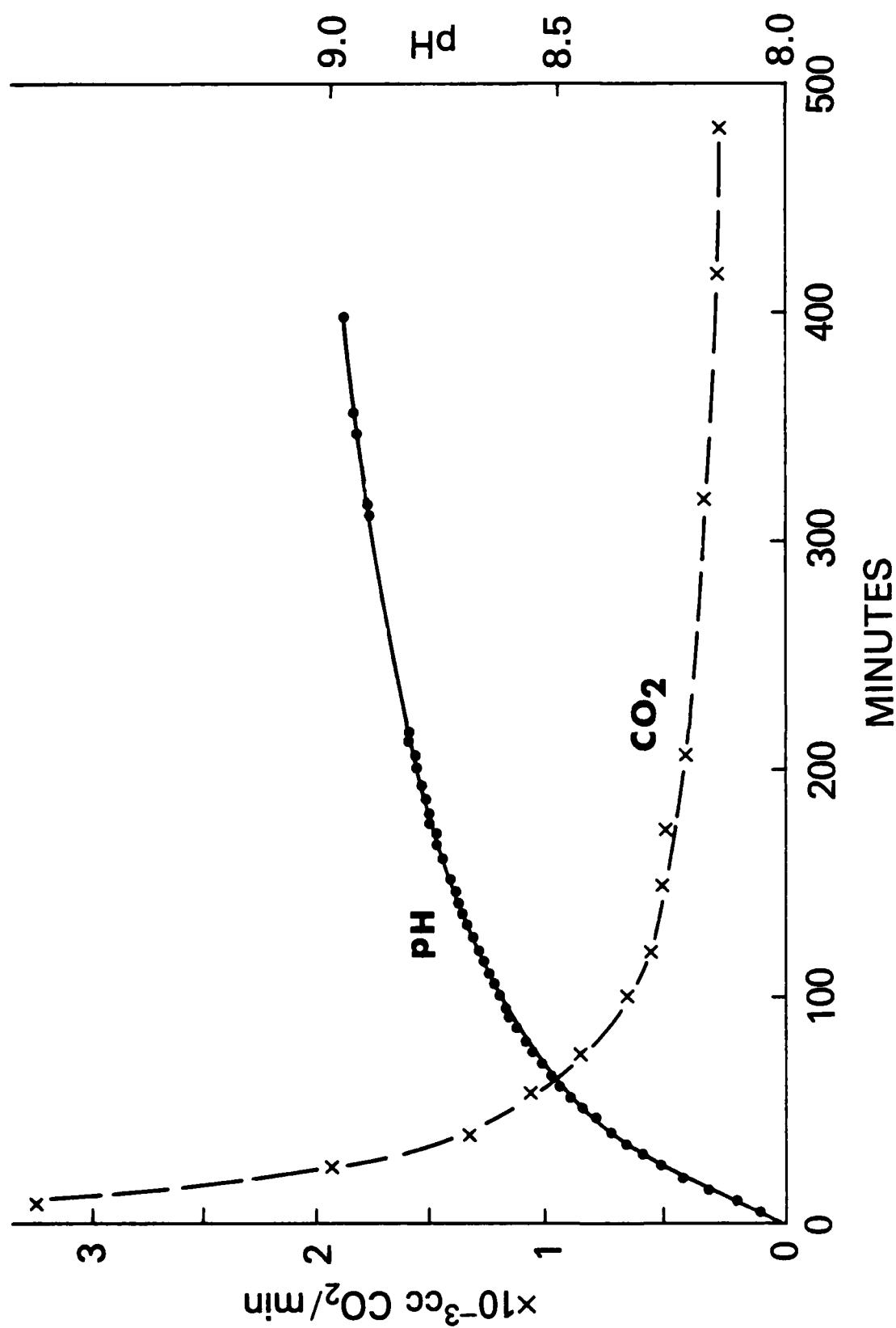


Fig. 12 — A plot of  $\text{CO}_2$  release, and rise in pH, of a 25% seawater solution from the time of introduction of 0 ppm  $\text{CO}_2$ . The solution had been in equilibrium with 346 ppm  $\text{CO}_2$ .

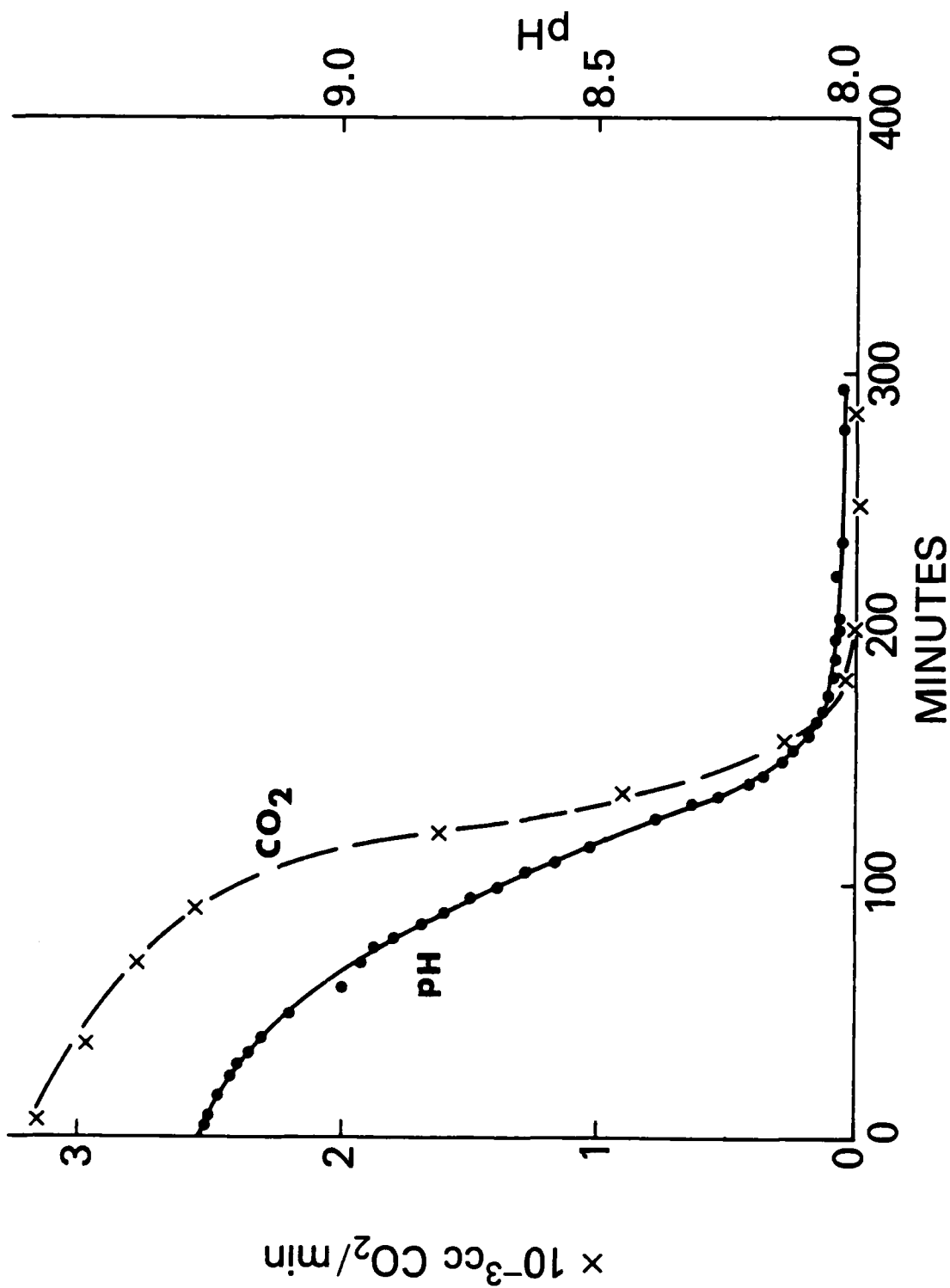


Fig. 13 — A plot of the CO<sub>2</sub> uptake, and decline in pH, of a 25% seawater solution from the time of introduction of air containing 346 ppm CO<sub>2</sub>. The solution had been aerated overnight with CO<sub>2</sub>-free air.

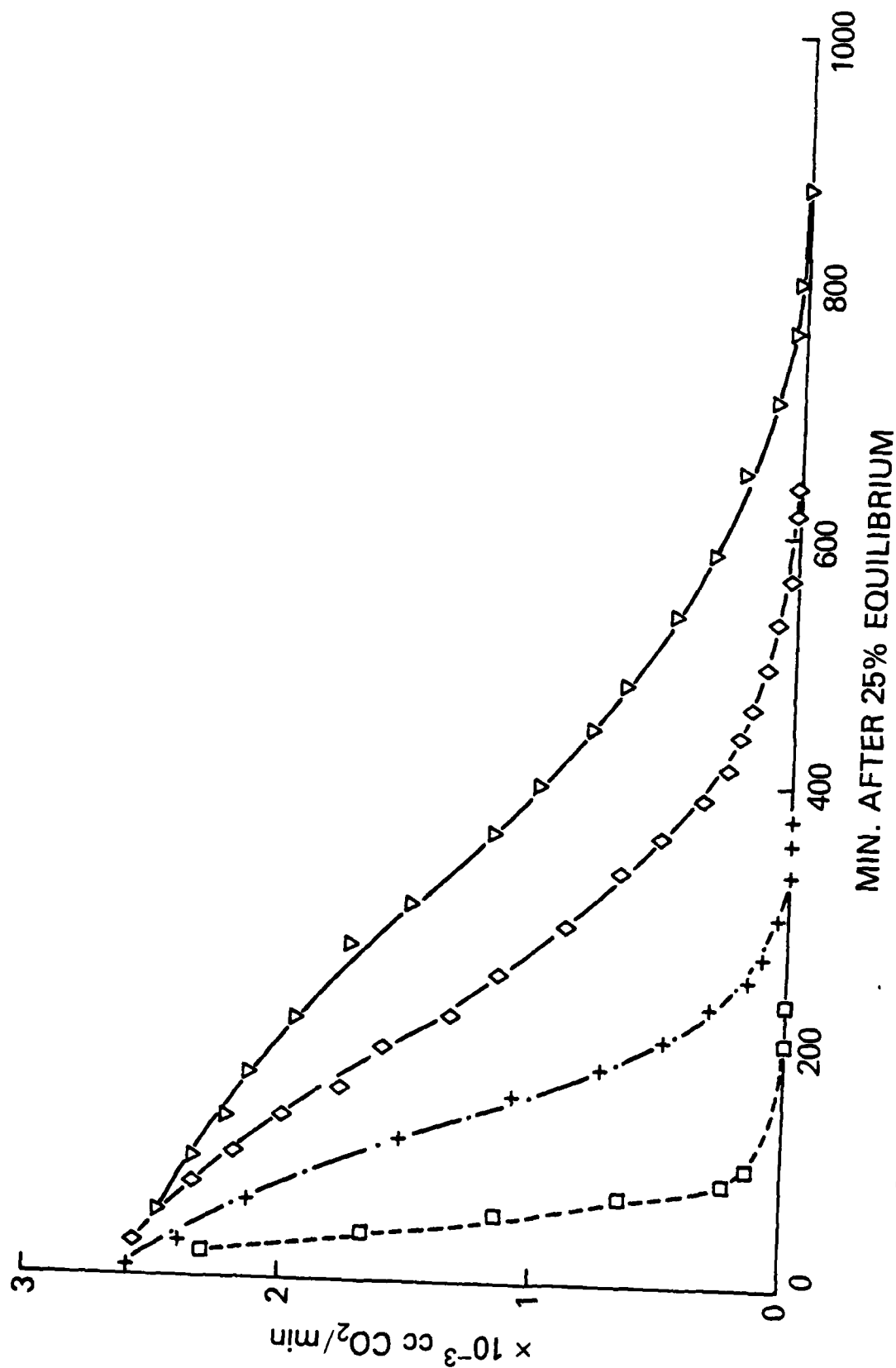


Fig. 14 - This plot illustrates the rates of  $\text{CO}_2$  uptake, with time, by various seawater solutions exposed to 346 ppm  $\text{CO}_2$  after having been initially almost at equilibrium with 0 ppm  $\text{CO}_2$ . The air flow rate was 10 cc/minute and the origin of the plots was taken to be an arbitrary rate of  $2.56 \times 10^{-3}$  cc  $\text{CO}_2$ /minute.

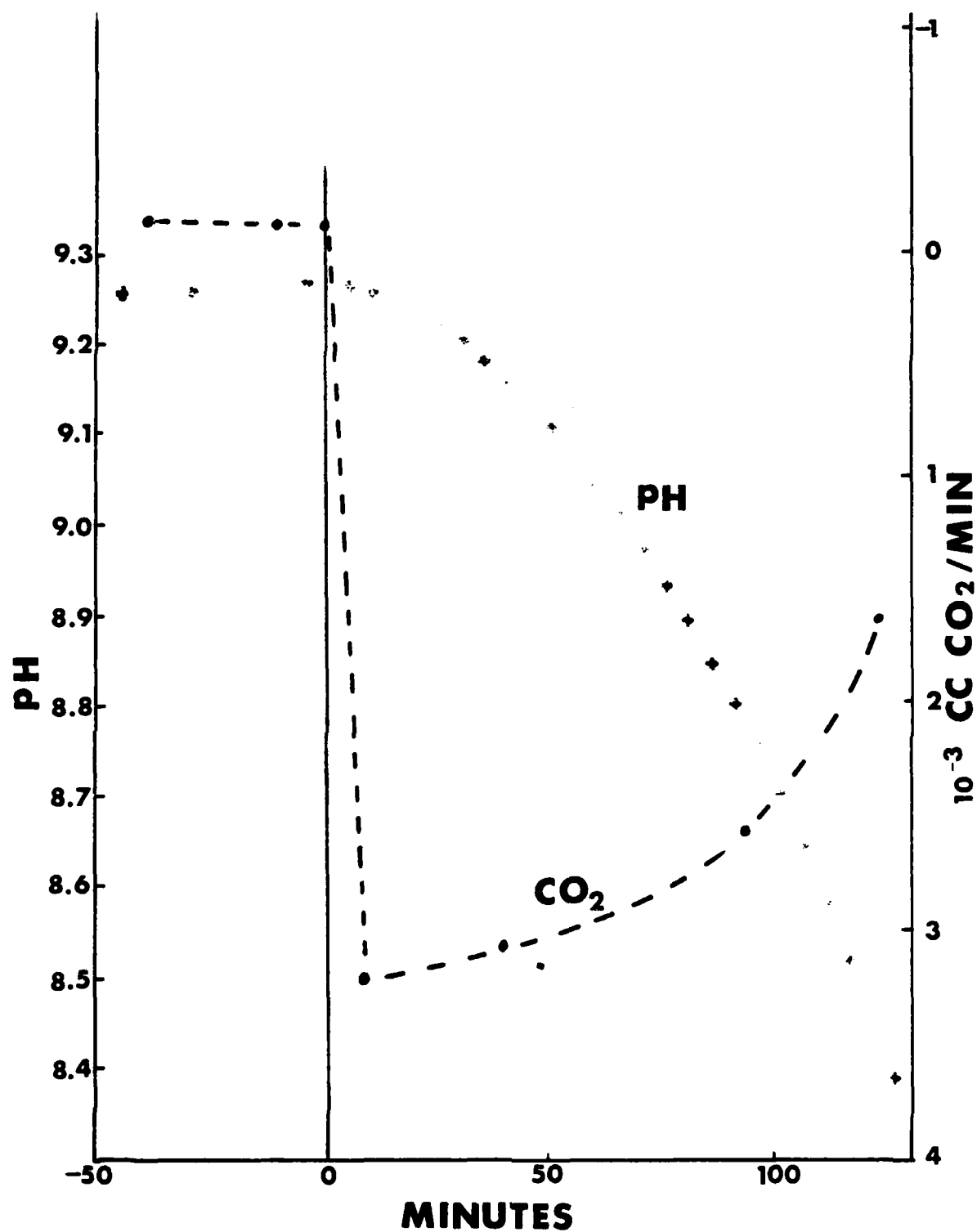


Fig. 15 — A plot showing the relationship between pH and CO<sub>2</sub>, before and after the substitution of air containing 346 ppm CO<sub>2</sub> for that containing 0 ppm CO<sub>2</sub>. The liquid was 25% seawater. Note the gradual change in pH vs the drastic initial absorption of CO<sub>2</sub>.

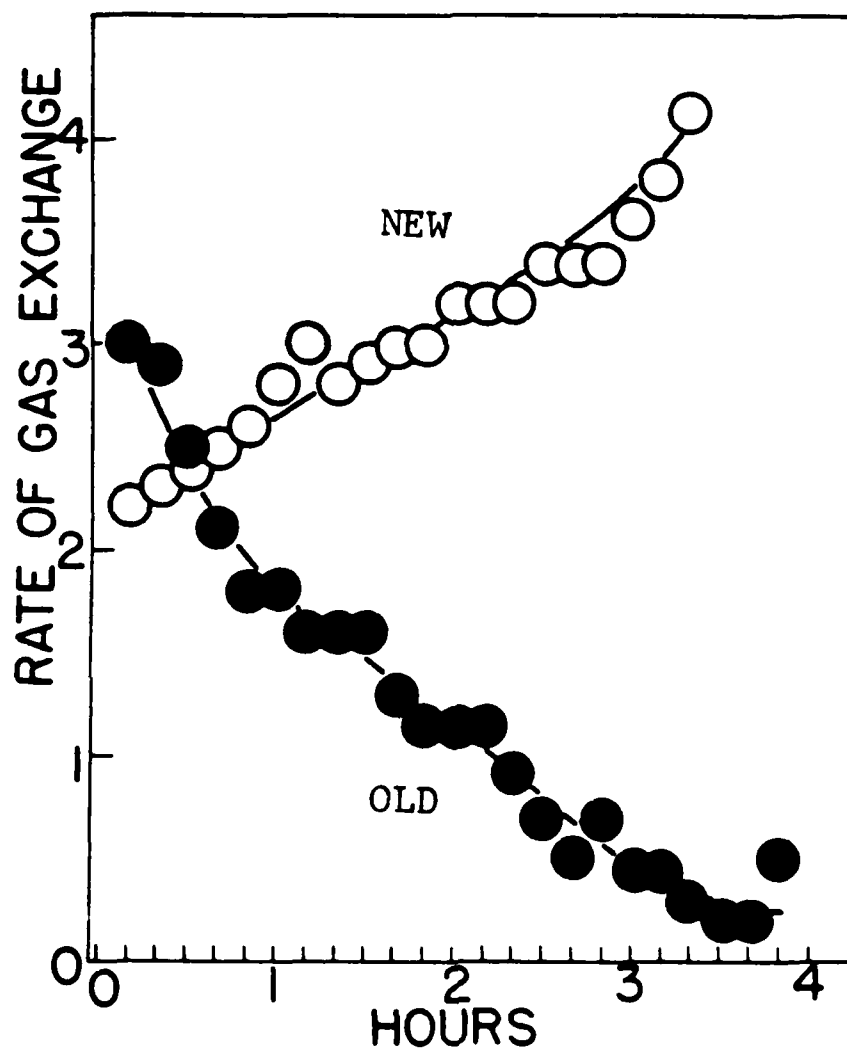


Fig. 16 — Rates of photosynthetic gas exchange (in relative units) for 10-minute intervals in synchronized 0- and 8-hour cells of *Chlorella*. Taken from Publication 1145, National Academy of Sciences, "On the Variability in the Activity of Photosynthetic Mechanisms." Printed with permission of the Academy.

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